US PAT NO: 5,780,597 [IMAGE AVAILABLE] L10: 4 of 7
TITLE: **Monoclonal antibodies** to cytotoxic lymphocyte maturation factor

ABSTRACT:
The present invention relates to antibodies which bind to a novel cytotoxic lymphocyte maturation factor. When bound to the cytotoxic lymphocyte maturation factor, the antibodies can neutralize bioactivity of the factor.

TITLE: **Monoclonal antibodies** to cytotoxic lymphocyte maturation factor

SUMMARY:

BSUM(10)

The present invention is a novel cytokine protein called Cytotoxic Lymphocyte Maturation Factor (CLMF) also called **IL-12** which is produced and synthesized by cells capable of secreting CLMF such as mammalian cells particularly human NC-37 B lymphoblastoid. . .

SUMMARY:

BSUM(18)
isolating . . . protein being Cytolytic Lymphocyte Maturation Factor (CLMF). CLMF is a 75 kilodalton (kDa) heterodimer comprised of two polypeptide subunits, a **40 kDa** subunit and a **35 kDa** subunit which are bonded together via one or more disulfide bonds.

SUMMARY:

BSUM(19)

The . . . T-cell growth, the substantially purified active protein, CLMF, obtained from the above described process, the isolated cloned genes encoding the **40 kDa** subunit and the **35 kDa** subunit, vectors containing these genes and host cells transformed with the vectors containing the genes.

SUMMARY:

BSUM(22)

Monoclonal antibodies prepared against a partially purified

```
Connecting via Winsock to STN

Trying 3106016892...Open

Welcome to STN International!  Enter x:x
LOGINID:ssspta1644axd
PASSWORD:
TERMINAL (ENTER 1, 2, 3, OR ?):2

* * * * * * * * * *  Welcome to STN International  * * * * * * * * * *

 NEWS  1         Web Page URLs for STN Seminar Schedule - N. America
 NEWS  2  Sep 17  IMSworld Pharmaceutical Company Directory name change
                 to PHARMASEARCH
 NEWS  3  Oct 09  Korean abstracts now included in Derwent World Patents
                 Index
 NEWS  4  Oct 09  Number of Derwent World Patents Index updates increased
 NEWS  5  Oct 15  Calculated properties now in the REGISTRY/ZREGISTRY File
 NEWS  6  Oct 22  Over 1 million reactions added to CASREACT
 NEWS  7  Oct 22  DGENE GETSIM has been improved
 NEWS  8  Oct 29  AAASD no longer available
 NEWS  9  Nov 19  New Search Capabilities  USPATFULL and USPAT2
 NEWS 10  Nov 19  TOXCENTER(SM) - new toxicology file now available on STN
 NEWS 11  Nov 29  COPPERLIT now available on STN
 NEWS 12  Nov 29  DWPI revisions to NTIS and US Provisional Numbers
 NEWS 13  Nov 30  Files VETU and VETB to have open access
 NEWS 14  Dec 10  WPINDEX/WPIDS/WPIX New and Revised Manual Codes for 2002
 NEWS 15  Dec 10  DGENE BLAST Homology Search
 NEWS 16  Dec 17  WELDASEARCH now available on STN
 NEWS 17  Dec 17  STANDARDS now available on STN
 NEWS 18  Dec 17  New fields for DPCI
 NEWS 19  Dec 19  CAS Roles modified
 NEWS 20  Dec 19  1907-1946 data and page images added to CA and CAplus
 NEWS 21  Jan 25  BLAST(R) searching in REGISTRY available in STN on the Web
 NEWS 22  Jan 25  Searching with the P indicator for Preparations
 NEWS 23  Jan 29  FSTA has been reloaded and moves to weekly updates
 NEWS 24  Feb 01  DKILIT now produced by FIZ Karlsruhe and has a new update
                 frequency
 NEWS 25  Feb 19  Access via Tymnet and SprintNet Eliminated Effective 3/31/02
 NEWS 26  Mar 08  Gene Names now available in BIOSIS

 NEWS EXPRESS  February 1 CURRENT WINDOWS VERSION IS V6.0d,
               CURRENT MACINTOSH VERSION IS V6.0a(ENG) AND V6.0Ja(JP),
               AND CURRENT DISCOVER FILE IS DATED 05 FEBRUARY 2002
 NEWS HOURS    STN Operating Hours Plus Help Desk Availability
 NEWS INTER    General Internet Information
 NEWS LOGIN    Welcome Banner and News Items
 NEWS PHONE    Direct Dial and Telecommunication Network Access to STN
 NEWS WWW      CAS World Wide Web Site (general information)

Enter NEWS followed by the item number or name to see news on that
specific topic.

  All use of STN is subject to the provisions of the STN Customer
  agreement.  Please note that this agreement limits use to scientific
  research.  Use for software development or design or implementation
  of commercial gateways or other similar uses is prohibited and may
  result in loss of user privileges and other penalties.

  * * * * * * * * * * * * * STN Columbus * * * * * * * * * * * * * * *


FILE 'HOME' ENTERED AT 14:05:10 ON 09 MAR 2002

=> file medline caplus embase biosis
COST IN U.S. DOLLARS                              SINCE FILE     TOTAL
                                                       ENTRY   SESSION
FULL ESTIMATED COST                                     0.15      0.15

FILE 'MEDLINE' ENTERED AT 14:05:23 ON 09 MAR 2002

FILE 'CAPLUS' ENTERED AT 14:05:23 ON 09 MAR 2002
USE IS SUBJECT TO THE TERMS OF YOUR STN CUSTOMER AGREEMENT.
PLEASE SEE "HELP USAGETERMS" FOR DETAILS.
COPYRIGHT (C) 2002 AMERICAN CHEMICAL SOCIETY (ACS)

FILE 'EMBASE' ENTERED AT 14:05:23 ON 09 MAR 2002
COPYRIGHT (C) 2002 Elsevier Science B.V.  All rights reserved.

FILE 'BIOSIS' ENTERED AT 14:05:23 ON 09 MAR 2002
COPYRIGHT (C) 2002 BIOLOGICAL ABSTRACTS INC.(R)

=> s (IL (1N) 12) or (Interleukin (1N) 12)
L1      27186 (IL (1N) 12) OR (INTERLEUKIN (1N) 12)

=> s l1 (P) antibod?
L2       4646 L1 (P) ANTIBOD?

=> s l1 (10N) antibod?
L3       1982 L1 (10N) ANTIBOD?

=> s l3 (10N) (p75)
L4         10 L3 (10N) (P75)

=> dup rem l4
PROCESSING COMPLETED FOR L4
L5            5 DUP REM L4 (5 DUPLICATES REMOVED)

=> dis l5 1-5 ibib abs kwic

L5   ANSWER 1 OF 5  BIOSIS  COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER:    2001:481591  BIOSIS
DOCUMENT NUMBER:     PREV200100481591
TITLE:               Antibodies against human IL-12.
AUTHOR(S):           Gately, Maurice Kent (1); Presky, David Howard
CORPORATE SOURCE:    (1) Parsippany, NJ USA
                     ASSIGNEE: Hoffman-La Roche Inc.
PATENT INFORMATION: US 6225117 May 01, 2001
SOURCE:              Official Gazette of the United States Patent and Trademark
```

Office Patents, (May 1, 20 ol. 1246, No. 1, pp. No Pagination. e-file.
ISSN: 0098-1133.

DOCUMENT TYPE: Patent
LANGUAGE: English

AB The present invention relates to novel **p75** heterodimer specific anti-human **IL-12 antibodies** that are characterized by a higher potency and greater efficacy in neutralizing human IL-12 bioactivity than known heterodimer specific **IL-12** monoclonal antibodies. The heterodimer specific **antibodies** recognize one or more epitopes of the human **IL-12 p75** heterodimer, but do not bind to the p40 subunit alone. The heterodimer specific IL-12 antibodies neutralize rhesus monkey IL-12 bioactivity with a potency similar to their potency for neutralizing human IL-12 bioactivity making them useful IL-12 antagonists for in vivo studies in the rhesus monkey.

AB The present invention relates to novel **p75** heterodimer specific anti-human **IL-12 antibodies** that are characterized by a higher potency and greater efficacy in neutralizing human IL-12 bioactivity than known heterodimer specific **IL-12** monoclonal antibodies. The heterodimer specific **antibodies** recognize one or more epitopes of the human **IL-12 p75** heterodimer, but do not bind to the p40 subunit alone. The heterodimer specific IL-12 antibodies neutralize rhesus monkey IL-12 bioactivity. . .

L5 ANSWER 2 OF 5 CAPLUS COPYRIGHT 2002 ACS

ACCESSION NUMBER: 2000:688272 CAPLUS
DOCUMENT NUMBER: 133:280563
TITLE: Human antibodies that bind human IL-12 and methods for producing
INVENTOR(S): Salfeld, Jochen G.; Roguska, Michael; Paskind, Michael; Banerjee, Subhashis; Tracey, Daniel E.; White, Michael; Kaymakcalan, Zehra; Labkovsky, Boris; Sakorafas, Paul; Friedrich, Stuart; Myles, Angela; Veldman, Geertruida M.; Venturini, Amy; Warne, Nicholas W.; Widom, Angela; Elvin, John G.; Duncan, Alexander R.; Derbyshire, Elaine J.; Carmen, Sara; Smith, Stephen; Holtet, Thor Las; Du, Fou Sarah L.
PATENT ASSIGNEE(S): Basf A.-G., Germany; Genetics Institute Inc.; et al.
SOURCE: PCT Int. Appl., 377 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2000056772	A1	20000928	WO 2000-US7946	20000324

W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
RW: GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

EP 1175446	A1	20020130	EP 2000-918396	20000324

R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO

PRIORITY APPLN. INFO.: US 1999-126603P P 19990325
WO 2000-US7946 W 20000324

AB Human antibodies, preferably recombinant human antibodies, that specifically bind to human interleukin-12 (hIL-12) are disclosed. Preferred antibodies have high affinity for hIL-12 and neutralize hIL-12 activity in vitro and in vivo . An antibody of the invention can be a full-length antibody or an antigen-binding portion thereof. The antibodies, or antibody portions, of the invention are useful for detecting hIL-12 and for inhibiting hIL-12 activity, e.g., in a human subject suffering from a disorder in which hIL-12 activity is detrimental. Nucleic acids, vectors and host cells for expressing the recombinant human antibodies of the invention, and methods of synthesizing the recombinant human antibodies, are also encompassed by the invention.

REFERENCE COUNT: 7 THERE ARE 7 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

IT Proteins, specific or class
RL: BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(**p75**, sol. TNF receptor; recombinant human **antibodies** that bind human **IL-12** for treatment of autoimmune diseases and inflammatory diseases)

L5 ANSWER 3 OF 5 CAPLUS COPYRIGHT 2002 ACS

ACCESSION NUMBER: 1999:487326 CAPLUS
DOCUMENT NUMBER: 131:129052
TITLE: Antibodies against human IL-12
INVENTOR(S): Gately, Maurcie Kent; Presky, David Howard
PATENT ASSIGNEE(S): F.Hoffmann-La Roche A.-G., Switz.
SOURCE: PCT Int. Appl., 47 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 9937682	A2	19990729	WO 1999-EP202	19990115

W: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
RW: GH, GM, KE, LS, MW, SD, SZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

AU 9925177	A1	19990115	AU 1999-25177	19990115
BR 9907743	A	20001017	BR 1999-7743	19990115
EP 1049717	A2	20001108	EP 1999-904780	19990115

R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, PT, IE, FI

JP 2002501085	T2	20020115	JP 2000-528602	19990115

```
US 6225117          B1    20010501        US        232522    19990119
ZA 9900452          A     19990723        ZA 1999-452         19990121
PRIORITY APPLN. INFO.:                    US 1998-72333    P  19980123
                                          WO 1999-EP202    W  19990115
```

AB The present invention relates to **p75** heterodimer specific anti-human **IL-12 antibodies** that are characterized by a higher potency and greater efficacy in neutralizing human IL-12 bioactivity than known heterodimer specific IL-12 monoclonal antibodies. The heterodimer specific **antibodies** recognize one or more epitopes of the human **IL-12 p75** heterodimer, but do not bind to the p40 subunit alone. The heterodimer specific IL-12 antibodies neutralize rhesus monkey IL-12 bioactivity with a potency similar to their potency for neutralizing human IL-12 bioactivity making them useful IL-12 antagonists. The monoclonal antibodies are therefore useful for diseases assocd. with aberrant Th1-type helper cell activity, e.g. multiple sclerosis, rheumatoid arthritis, autoimmune diabetes mellitus, Crohn's disease and ulcerative colitis.

AB The present invention relates to **p75** heterodimer specific anti-human **IL-12 antibodies** that are characterized by a higher potency and greater efficacy in neutralizing human IL-12 bioactivity than known heterodimer specific IL-12 monoclonal antibodies. The heterodimer specific **antibodies** recognize one or more epitopes of the human **IL-12 p75** heterodimer, but do not bind to the p40 subunit alone. The heterodimer specific IL-12 antibodies neutralize rhesus monkey IL-12 bioactivity with a potency similar to their potency for neutralizing human IL-12 bioactivity making them useful IL-12 antagonists. The monoclonal antibodies are therefore useful for diseases assocd. with aberrant Th1-type helper cell activity, e.g. multiple sclerosis, rheumatoid arthritis, autoimmune diabetes mellitus, Crohn's disease and ulcerative colitis.

```
L5  ANSWER 4 OF 5     MEDLINE                          DUPLICATE 1
ACCESSION NUMBER:     1999057032      MEDLINE
DOCUMENT NUMBER:      99057032    PubMed ID: 9842898
TITLE:                Defective Th1 and Th2 cytokine synthesis in the T-T cell
                      presentation model for lack of CD40/CD40 ligand
                      interaction.
AUTHOR:               De Vita L; Accapezzato D; Mangino G; Morrone S; Santilio I;
                      Casciaro M A; Fava D; Bruno G; Del Prete G; Santoni A;
                      Barnaba V
CORPORATE SOURCE:     Fondazione Andrea Cesalpino, Istituto di Clinica Medica,
                      Universita di Roma La Sapienza, Rome, Italy.
SOURCE:               EUROPEAN JOURNAL OF IMMUNOLOGY, (1998 Nov) 28 (11) 3552-63.
                      Journal code: EN5; 1273201. ISSN: 0014-2980.
PUB. COUNTRY:         GERMANY: Germany, Federal Republic of
                      Journal; Article; (JOURNAL ARTICLE)
LANGUAGE:             English
FILE SEGMENT:         Priority Journals
ENTRY MONTH:          199812
ENTRY DATE:           Entered STN: 19990115
                      Last Updated on STN: 19990115
                      Entered Medline: 19981217
```

AB In this study, T or NK cell clones used as antigen-presenting cells (T- or NK-APC) were shown to be significantly less efficient than professional APC in inducing Th1 and Th2 cytokines by antigen-specific T cell clones. This phenomenon was not related to a limited engagement of TCR by T-APC, since comparable thresholds of TCR down-regulation were shown when antigen was presented by either T-APC or professional APC. Rather, the stimulatory T-APC weakness was due to their inability, because they are CD40-, to provide the appropriate co-stimuli to responder T cells both indirectly via IL-12, and partially via direct CD40L triggering on T cells. Indeed, the simultaneous addition of IL-12 and reagents directly engaging CD40L on responder T cells restored T cell cytokine synthesis when antigen was presented by T-APC. In addition, either IL-12 production or blocking of T cell cytokine synthesis by anti-**IL-12 p75** **antibodies** was evident only when professional APC were used in our antigen-specific system. The down-regulation of cytokine synthesis in the system of T-T cell presentation could represent a novel mechanism of immune regulation, which may intervene to switch off detrimental Th1- or Th2-mediated responses induced by antigen presentation among activated T cells infiltrating inflamed tissues.

AB . . . synthesis when antigen was presented by T-APC. In addition, either IL-12 production or blocking of T cell cytokine synthesis by anti-**IL-12 p75 antibodies** was evident only when professional APC were used in our antigen-specific system. The down-regulation of cytokine synthesis in the system. . .

```
L5  ANSWER 5 OF 5     MEDLINE                          DUPLICATE 2
ACCESSION NUMBER:     1998043635      MEDLINE
DOCUMENT NUMBER:      98043635    PubMed ID: 9373257
TITLE:                Protective effect of a single interleukin-12 (IL-12)
                      predose against the toxicity of subsequent chronic IL-12 in
                      mice: role of cytokines and glucocorticoids.
AUTHOR:               Sacco S; Heremans H; Echtenacher B; Buurman W A; Amraoui Z;
                      Goldman M; Ghezzi P
CORPORATE SOURCE:     Laboratory of Neuroimmunology, "Mario Negri" Institute for
                      Pharmacological Research, Milano, Italy.
SOURCE:               BLOOD, (1997 Dec 1) 90 (11) 4473-9.
                      Journal code: A8G; 7603509. ISSN: 0006-4971.
PUB. COUNTRY:         United States
                      Journal; Article; (JOURNAL ARTICLE)
LANGUAGE:             English
FILE SEGMENT:         Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH:          199712
ENTRY DATE:           Entered STN: 19980109
                      Last Updated on STN: 19980109
                      Entered Medline: 19971223
```

AB The mechanisms of interleukin-12 (IL-12) toxicity were studied in mice using a schedule (murine rIL-12, 400 ng/mouse, intraperitoneally [IP] once daily for 5 days) that markedly reduced body weight and food intake. On day 5, IL-12-treated mice had elevated serum and spleen IFN-gamma and tumor necrosis factor (TNF). Serum sTNFR-P75 and corticosterone (CS) were also elevated. **IL-12** toxicity was partially prevented by anti-IFN-gamma **antibodies** or dexamethasone (DEX). A pre-dose of IL-12 (200 ng/mouse on day -14) completely prevented the toxicity of subsequent IL-12. The IL-12 predose also inhibited IL-12-induced IFN-gamma levels, but did not modify IL-12-induced CS, TNF or sTNFR-P75. A protective effect was observed with a predose of lipopolysaccharide (LPS) or murine recombinant (r)IL-10. The protective effect of the IL-12 predose was reduced by coadministration of anti-IFN-gamma, but a predose of murine rIFN-gamma was not protective,

suggesting that IFN-gamma is necessary but not sufficient for the
protective effect of IL-12. The IL-12 predose specifically protected
against IL-12 toxicity and did not modify LPS toxicity. These data
indicate that IL-12 can induce tolerance to its own toxicity, probably
through a downregulation of IL-12-induced IFN-gamma but independently of
endogenous glucocorticoids. IFN-gamma, and possibly IL-10, might be
important in this tolerance.

AB . . . and food intake. On day 5, IL-12-treated mice had elevated serum
and spleen IFN-gamma and tumor necrosis factor (TNF). Serum sTNFR-
P75 and corticosterone (CS) were also elevated. **IL-**
12 toxicity was partially prevented by anti-IFN-gamma
antibodies or dexamethasone (DEX). A pre-dose of IL-12 (200
ng/mouse on day -14) completely prevented the toxicity of subsequent
IL-12. The. . .

=> dis his

(FILE 'HOME' ENTERED AT 14:05:10 ON 09 MAR 2002)

FILE 'MEDLINE, CAPLUS, EMBASE, BIOSIS' ENTERED AT 14:05:23 ON 09 MAR 2002

L1 27186 S (IL (1N) 12) OR (INTERLEUKIN (1N) 12)
L2 4646 S L1 (P) ANTIBOD?
L3 1982 S L1 (10N) ANTIBOD?
L4 10 S L3 (10N) (P75)
L5 5 DUP REM L4 (5 DUPLICATES REMOVED)

=> s l5 (P) neutraliz?
PROXIMITY OPERATOR LEVEL NOT CONSISTENT WITH
FIELD CODE - 'AND' OPERATOR ASSUMED 'L26 (P) NEUTRALIZ'
L6 2 L5 (P) NEUTRALIZ?

=> dis l6 1-2 ibib abs kwic

L6 ANSWER 1 OF 2 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1999:487326 CAPLUS
DOCUMENT NUMBER: 131:129052
TITLE: Antibodies against human IL-12
INVENTOR(S): Gately, Maurcie Kent; Presky, David Howard
PATENT ASSIGNEE(S): F.Hoffmann-La Roche A.-G., Switz.
SOURCE: PCT Int. Appl., 47 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 9937682	A2	19990729	WO 1999-EP202	19990115
W: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, GM, KE, LS, MW, SD, SZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG				
AU 9925177	A1	19990115	AU 1999-25177	19990115
BR 9907743	A	20001017	BR 1999-7743	19990115
EP 1049717	A2	20001108	EP 1999-904780	19990115
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, PT, IE, FI				
JP 2002501085	T2	20020115	JP 2000-528602	19990115
US 6225117	B1	20010501	US 1999-232522	19990119
ZA 9900452	A	19990723	ZA 1999-452	19990121
PRIORITY APPLN. INFO.:			US 1998-72333 P	19980123
			WO 1999-EP202 W	19990115

AB The present invention relates to **p75** heterodimer specific
anti-human **IL-12 antibodies** that are
characterized by a higher potency and greater efficacy in
neutralizing human IL-12 bioactivity than known heterodimer
specific IL-12 monoclonal antibodies. The heterodimer specific
antibodies recognize one or more epitopes of the human **IL**
-12 p75 heterodimer, but do not bind to the p40
subunit alone. The heterodimer specific IL-12 antibodies
neutralize rhesus monkey IL-12 bioactivity with a potency similar
to their potency for **neutralizing** human IL-12 bioactivity making
them useful IL-12 antagonists. The monoclonal antibodies are therefore
useful for diseases assocd. with aberrant Th1-type helper cell activity,
e.g. multiple sclerosis, rheumatoid arthritis, autoimmune diabetes
mellitus, Crohn's disease and ulcerative colitis.

AB The present invention relates to **p75** heterodimer specific
anti-human **IL-12 antibodies** that are
characterized by a higher potency and greater efficacy in
neutralizing human IL-12 bioactivity than known heterodimer
specific IL-12 monoclonal antibodies. The heterodimer specific
antibodies recognize one or more epitopes of the human **IL**
-12 p75 heterodimer, but do not bind to the p40
subunit alone. The heterodimer specific IL-12 antibodies
neutralize rhesus monkey IL-12 bioactivity with a potency similar
to their potency for **neutralizing** human IL-12 bioactivity making
them useful IL-12 antagonists. The monoclonal antibodies are therefore
useful for diseases assocd. with aberrant Th1-type helper cell activity,
e.g. multiple sclerosis, rheumatoid arthritis, autoimmune diabetes
mellitus, Crohn's disease and ulcerative colitis.

L6 ANSWER 2 OF 2 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 2001:481591 BIOSIS
DOCUMENT NUMBER: PREV200100481591
TITLE: Antibodies against human IL-12.
AUTHOR(S): Gately, Maurice Kent (1); Presky, David Howard
CORPORATE SOURCE: (1) Parsippany, NJ USA
ASSIGNEE: Hoffman-La Roche Inc.
PATENT INFORMATION: US 6225117 May 01, 2001
SOURCE: Official Gazette of the United States Patent and Trademark
Office Patents, (May 1, 2001) Vol. 1246, No. 1, pp. No
Pagination. e-file.
ISSN: 0098-1133.
DOCUMENT TYPE: Patent
LANGUAGE: English

AB The present invention relates to novel **p75** heterodimer specific
anti-human **IL-12 antibodies** that are
characterized by a higher potency and greater efficacy in

-n heterodimer
erodimer
topes of the human

L-12 antibodies
th a potency similar
bioactivity making
dies in the rhesus monkey.
terodimer specific

efficacy in
wn heterodimer
erodimer
topes of the human

L-12 antibodies
th a potency similar
bioactivity making
dies in the rhesus monkey.

CAL ABSTRACTS INC.

ky, David

States Patent and Trademark
Vol. 1246, No. 1, pp. No

terodimer specific

efficacy in neutralizing
r

or more epitopes

dimer specific IL-

r potency for
hem
in the

IL-

sky, David

Switz.

TION NO. DATE
-------- --------
-EP202   19990115
Y CA, CH, CN, CU, CZ, DE,
U ID, IL, IN, IS, JP, KE,
U, LV, MD, MG, MK, MN, MW,
G, SI, SK, SL, TJ, TM, TR,
Y, KG, KZ, MD, RU, TJ, TM
T BE, CH, CY, DE, DK, ES,
I SE, BF, BJ, CF, CG, CI,
G
-25177   19990115
-7743   19990115
-904780   19990115
T, LI, LU, NL, SE, PT, IE, FI
-528602   19990115
-232522   19990119
-452   19990121
333   P 19980123
202   W 19990115
mer specific

efficacy in neutralizing
r

or more epitopes

odimer specific IL-

12 bioactivity with a potency similar to potency for neutralizing human **IL-12** bioactivity making them useful **IL-12** antagonists. The monoclonal **antibodies** are therefore useful for diseases assocd. with aberrant Th1-type helper cell activity, e.g. multiple sclerosis, rheumatoid arthritis, autoimmune diabetes mellitus, Crohn's disease and ulcerative colitis.

```
L11  ANSWER 3 OF 3     MEDLINE                                DUPLICATE 1
ACCESSION NUMBER:    96163540     MEDLINE
DOCUMENT NUMBER:     96163540   PubMed ID: 8576576
TITLE:               Characterization of anti-mouse IL-12
                     monoclonal antibodies and measurement of mouse
                     IL-12 by ELISA.
AUTHOR:              Wilkinson V L; Warrier R R; Truitt T P; Nunes P;
                     Gately M K; Presky D H
CORPORATE SOURCE:    Department of Inflammation/Autoimmune Diseases, Roche
                     Research Center, Hoffmann-La Roche Inc., Nutley, NJ 07110,
                     USA.
SOURCE:              JOURNAL OF IMMUNOLOGICAL METHODS, (1996 Jan 16) 189 (1)
                     15-24.
                     Journal code: IFE; 1305440. ISSN: 0022-1759.
PUB. COUNTRY:        Netherlands
                     Journal; Article; (JOURNAL ARTICLE)
LANGUAGE:            English
FILE SEGMENT:        Priority Journals
ENTRY MONTH:         199603
ENTRY DATE:          Entered STN: 19960321
                     Last Updated on STN: 19960321
                     Entered Medline: 19960313
```

AB Enzyme-linked immunosorbent assays (ELISAs) capable of quantitatively measuring pg/ml amounts of mouse **IL-12** (moIL-12) were developed as an alternative to the current bioassay procedure used for the measurement of moIL-12. A panel of 40 rat anti-moIL-12 monoclonal **antibodies** were identified and tested for their ability to bind 125I-moIL-12. Two of the MAbs, 2B5 and 9A5, were able to capture 125I-moIL-12 in the presence of unlabelled moIL-12 p35 and moIL-12 p40, suggesting specificity for the moIL-12 **p75** heterodimer. Western blot analysis confirmed that MAb 9A5 specifically recognized only moIL-12 **p75**. Using MAb 9A5, and an additional anti-moIL-12 p40 MAb 5D9, we developed quantitative ELISAs for the specific detection of moIL-12 **p75** and p40, respectively. These ELISAs detect moIL-12 with a sensitivity of 40 pg/ml. Whereas the p40 ELISA detected three forms of moIL-12 (p40 monomer, p40 homodimer, and the heterodimer), the **p75** ELISA only detected moIL-12 heterodimer. Neither of these assays crossreacted with a panel of additional cytokines. The levels of moIL-12 measured by the **p75** ELISA and the bioassay were directly compared and found to correlate well. Therefore, the **p75** ELISA represents an alternative to the bioassay for the measurement of moIL-12.

```
=> end
ALL L# QUERIES AND ANSWER SETS ARE DELETED AT LOGOFF
LOGOFF? (Y)/N/HOLD:y
COST IN U.S. DOLLARS                              SINCE FILE     TOTAL
                                                       ENTRY   SESSION
FULL ESTIMATED COST                                    49.54     49.69

DISCOUNT AMOUNTS (FOR QUALIFYING ACCOUNTS)        SINCE FILE     TOTAL
                                                       ENTRY   SESSION
CA SUBSCRIBER PRICE                                    -2.48     -2.48

STN INTERNATIONAL LOGOFF AT 14:13:25 ON 09 MAR 2002
```

Harbor Laboratory, Cold Spring Harbor, N.Y., (1988).

CLAIMS:

CLMS(1)

What . . .

disease, comprising administering to a subject diagnosed with an established colitis from an inflammatory bowel disease an amount of an **antibody** to interleukin-12 effective in reducing the colitis-inducing effect of interleukin-12, thereby treating the inflammatory response of an established colitis.

CLAIMS:

CLMS(3)

3. . . . interferon-gamma by lamina propria cells is assayed according to a method selected from the group consisting of ELISA, reverse transcriptase-polymerase **chain** reaction and ELISPOT.

CLAIMS:

CLMS(6)

6. The method of claim 2, wherein the animal is a **mouse**.

CLAIMS:

CLMS(7)

7. . . .
a colitis; and
c) assaying the lamina propria cells of the animal for an amount of secretion of interferon-gamma, whereby a **lack** of increase in the amount of interferon-gamma secreted by the lamina propria cells of the animal as compared to an. . .

CLAIMS:

CLMS(8)

8. . . . interferon-gamma by lamina propria cells is assayed according to a method selected from the group consisting of ELISA, reverse transcriptase-polymerase **chain** reaction and ELISPOT.

CLAIMS:

CLMS(9)

9. The method of claim 7, wherein the animal is a **mouse**.

TITLE:	Methods of treating established colitis using **antibodies** against IL-12		
US PAT NO:	5,853,697 [IMAGE AVAILABLE]	DATE ISSUED:	Dec. 29, 1998
APPL-NO:	08/547,979	DATE FILED:	Oct. 25, 1995

US PAT NO:	5,547,854 [IMAGE AVAILABLE]	L7: 2 of 2
TITLE:	DNA encoding a receptor for Mullerian inhibitory substance, misrl, and corresponding vectors, cells, probes, and recombinant methods	

ABSTRACT:

Isolated DNAs (e.g., cDNAs or genomic fragments) encoding MIS receptors, inhibin receptors, bone morphogenic protein receptors, or other novel members of the TGF-.beta. family of receptors, or soluble, ligand-binding fragments thereof; vectors or cells which contain such DNAs; and substantially pure polypeptides encoded by such DNAs, whether produced by expression of the isolated DNAs, by isolation from natural sources, or by chemical synthesis.

SUMMARY:

BSUM(3)

Mullerian . . . al., In Vitro Cellular and Developmental Biol. 25:213-216, 1989); more recently, the rat (Haqq et al., Genomics 12:665-9, 1992) and **mouse** (Munsterberg and Lovell-Badge, Development 13:613-624, 1991) genes have also been cloned. Overexpression of MIS in **transgenic** female **mice** caused regression of Mullerian ducts and seminiferous tubular differentiation (Behringer et al., Nature 345:167-70, 1991). Several patients with Retained Mullerian Duct Syndrome were found to have point **mutations** in the MIS gene (Knebelman et al., Proc. Natl. Acad. Sci. 88:3767-3771, 1991), which has been localized to the short arm of chromosome 19 (Cohen-Hagenaur et al., Cytogenet. Cell. Genet. 44:2-6, 1987). In **mice**, the MIS gene is located on chromosome 10 (King et al., Genomics 11:273-283, 1991).

SUMMARY:

BSUM(7)

Inhibin, . . . USA 83:5849, 1986; Esch et al., Mol. Endocrinol. 1:388, 1987). Inhibin was shown at that time to be a glycoprotein **heterodimer** composed of an alpha-**chain** and one of two distinct beta-**chains** (beta-A, beta-B) (Mason et al., Biochem. Biophys. Res. Comun. 135:957, 1986). The alpha **chain** is processed from an initial species of 57 kDa to form an 18 kDa carboxyl-terminal peptide, while the mature beta **chain** of 14 kDa is cleaved from the carboxyl-terminus of a 62 kDa precursor, which would then account for the biologically. . . of 32-120 kDa, however, have been isolated as well (Miyamoto et al., Biochem. Biophys. Res. Commun. 136:1103-9, 1986). In addition, beta-**chain** dimers (beta-A/beta-A or beta-A/beta-B) which selectively stimulate FSH secretion from the pituitary have been identified and are called activin A. . .

SUMMARY:

BSUM(9)

Most . . . data that exist concerning serum inhibin levels in humans have been obtained using a heterologous radioimmunoassay comprised of a polyclonal **antibody** to purified, intact bovine inhibin and radiolabeled 32 kDa bovine inhibin (McLachlan et al., Mol. Cell. Endocrinol. 46:175-85, 1986). Such. . .

SUMMARY:

BSUM(11)

The . . . inhibin receptors, and bone morphogenesis protein (BMP) receptors; these receptors are, e.g., those of a mammal such as a rat, **mouse**, rabbit, guinea pig, hamster, cow, pig, horse, goat, sheep, or human. The invention also includes vectors (e.g., plasmids, phage, or. . .

SUMMARY:

BSUM(15)

As . . . the amount of MIS or inhibin activity in the sample. They can also be used to generate monoclonal or polyclonal **antibodies** specific for (i.e., capable of forming an immune complex with) such receptors, which **antibodies** would be useful in a method for detecting the presence of an MIS or inhibin receptor in a biological sample such as serum or tumor cells. Such a method would include the steps of (1) contacting the **antibody** with a biological sample suspected of containing an MIS or inhibin receptor, and (2) detecting immune complex formation between the **antibody** and a component of the biological sample, wherein such immune complex formation is indicative of the presence of such a receptor in the sample. Furthermore, such **antibodies** can be linked to a cytotoxic agent, thereby forming an immunotoxin useful for targeting and killing or disabling cells bearing. . .

DRAWING DESC:

DRWD(16)

FIG. . . . E21 samples are from tests collected at days 15 and 21 of gestation, respectively; P7, P14, P20, P24, P27, P30, **P35**, **P40**, and P60 samples are all from postnatal animals. (10 .mu.g of total RNA per lane; 4 day exposure.)

DETDESC:

DETD(8)

(a) . . . detection of binding to the transfected cells (which presumably bear the recombinant receptor on their surfaces), or alternatively a labelled **antibody** specific for the ligand can be used to indicate whether or not the cells have bound ligand. Binding of the. . . U.S. Pat. No. 5,047,336 (herein incorporated by reference), and purified by means of an affinity column using an anti-MIS monoclonal **antibody**, such as disclosed in Donahoe et al., U.S. Pat. No. 4,792,601 (herein incorporated by reference). The purified holo MIS is. . .

DETDESC:

DETD(10)

(c) . . . are transfected with the putative receptor DNA of the invention, and used, in accordance with standard procedures, to generate monoclonal **antibodies** which can differentiate between such transfected cells and identical but untransfected cells. These monoclonal **antibodies** are then labelled and used in immunohistochemical analysis of given tissues, in order to determine what tissues normally express the. . .

DETDESC:

DETD(11)

(d) Monoclonal **antibodies** raised as described above could also be used in a competitive binding assay. A given tissue sample which, by virtue. . . occurring MIS or inhibin receptors could be employed in a competitive binding assay with either labelled ligand and excess unlabelled **antibody** raised against the putative receptor (as described above), or labelled **antibody** and excess unlabelled ligand. Evidence that the ligand and the **antibody** compete for the same binding sites would support the conclusion that the putative receptor was indeed an MIS, inhibin, or. . .

DETDESC:

DETD(12)

(d) . . . known to be affected by MIS, inhibin, BMP, or another candidate ligand, including both normal and disease-state tissues, and the **lack** of detectible expression in other tissues known to be insensitive to the candidate ligand, is evidence that the putative receptor. . .

DETDESC:

DETD(14)

The . . . cell. Purified receptor protein, or cells or membrane preparations bearing the receptor, may be used to generate monoclonal or polyclonal **antibodies** specific for the given receptor, which **antibodies** can be employed in assays for detecting the presence or the amount of such receptor in biological samples such as. . . and it is postulated that the growth of other tumor types may be similarly reduced by inhibin or BMP. The **antibodies** of the invention would therefore be useful for identifying candidate tumors likely to respond to therapy with MIS, inhibin, BMP, or agonists or antagonists thereof. The receptor polypeptides of the invention, and their respective **antibodies**, could be used as receptor agonists or antagonists in the management of relevant clinical disorders. The **antibodies** can also be used as the targeting means for directing cytotoxic agents to cells (such as tumor cells) bearing the. . .

DETDESC:

DETD(16)

Recombinant . . . example, by means of a sandwich assay utilizing the recombinant receptor protein fixed to a solid support, and labelled anti-ligand **antibody**. Where the ligand being measured is MIS, it may be desireable to include plasmin or an MIS-specific protease in the. . .

DETDESC:

DETD(23)
Polymerase **chain** reaction (PER) using consensus primers.

DETDESC:

DETD(24)

The DNA sequence of the cDNA encoding a **murine** activin receptor (Mathews and Vale, Cell 65:973-982, 1991) was compared to that of certain related cDNAs: human and porcine TGF-.beta.. . .

DETDESC:

DETD(26)

The oligonucleotides were synthesized with an Applied Biosystems 391 DNA synthesizer, and used as primers for polymerase **chain** reaction (PCR)-based selection from a 14.5 day rat urogenital ridge COS cell expression cDNA library. PCR was carried out in. . .

DETDESC:

DETD(44)

Recombinant . . . ml immunoaffinity column was constructed using approximately 50 mg of the protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.) purified **mouse** monoclonal anti-human rhMIS **antibody** (Hudson et al., J. Clin. Endocrinol. Metab. 70:16-22, 1990) covalently attached to Affigel-10 agarose resin (BioRad Laboratories, Richmond, Calif.). The. . .

TITLE:	DNA encoding a receptor for Mullerian inhibitory substance, misr1, and corresponding vectors, cells, probes, and recombinant methods		
US PAT NO:	5,547,854 [IMAGE AVAILABLE]	DATE ISSUED:	Aug. 20, 1996
APPL-NO:	08/317,847	DATE FILED:	Oct. 4, 1994
REL-US-DATA:	Continuation of Ser. No. 29,673, Mar. 11, 1993, abandoned, which is a continuation-in-part of Ser. No. 853,396, Mar. 18, 1992, abandoned.		

continuation-in-part of Ser. No. 320,471, Mar. 8, 1989, Pat. No. 5,155,020.

US PAT NO: 5,831,007 [IMAGE AVAILABLE] L10: 3 of 7
TITLE: Human receptor for interleukin-12

ABSTRACT:
This invention relates to substantially pure Interleukin-12 receptor cDNAs and protein and uses therefore. The Interleukin-12 receptor is shown to be a member of the cytokine receptor superfamily and has a high homology to human gp130.

SUMMARY:

BSUM(4)

Interleukin-12 (**IL-12**), formerly known as cytotoxic lymphocyte maturation factor or natural killer cell stimulatory factor, is a 75-KDa heterodimeric cytokine composed of disulfide-bonded **40-KDa** (p40) and **35-KDa** (p35) subunits that has pleiotropic activities including stimulation of the proliferation of activated T and NK cells (Gately, M. K.,. . .

SUMMARY:

BSUM(5)

The biological activity of **IL-12** is mediated by the binding of the **IL-12** molecules to cell surface, or plasma membrane, receptors on activated T- and NK cells; however, the contributions of the individual subunits, p35 and p40, to receptor binding and signal transduction remain unknown. Studies with labeled **IL-12** have shown that this binding occurs in a specific and saturable manner. **IL-12** delivers a signal to target cells through a receptor that was initially characterized on PHA-activated CD4+ and CD8+ T-cells and. . . NK- and myelomonocytic lineages only identified a single CD4+, IL-2 dependent human T-cell line (Kit 225) that constitutively expresses the **IL-12** receptor and responds to **IL-12** (B. Desai, et al., 1992, J. Immunol., 148:3125; B. Desai, et al., 1993, J. Immunol. 150:207A). Freshly prepared PHA-activated PBMC and the Kit 225 cell line thus represent two convenient cell sources to study the biochemistry of the functional **IL-12** receptor; there may be others. Equilibrium binding experiments with .sup.125 1-labeled **IL-12** showed that i) PHA-activated PBMC express several thousand **IL-12** receptors which show 3 classes of affinities: high=5-20 pM, intermediate=50-200 pM and low =2-6 nM; ii) **IL-12** receptor expression on PBMC is upregulated by mitogen or IL-2 stimulation; and iii) the **IL-12** receptor upregulation correlates with the ability of the cells to proliferate in response to **IL-12** (R. Chizzonite, et al., 1992, J. Immunol., 148:3117; B. Desai, et al., 1992, J. Immunol., 148:3125). It was not clear at this point whether the biologically functional **IL-12** receptor consists of one or more subunits. Affinity crosslinking of labeled **IL-12** to activated PBMC demonstrated the size of the cell surface **IL-12** binding protein(s) under nonreducing conditions to be in the range of about 150 KDa to about 200 KDa. Additional affinity crosslinking and immunoprecipitation experiments with unlabeled **IL-12** bound to .sup.125 I-surface labeled activated PBMC identified an **IL-12** binding protein that under reducing conditions had a size of about 110 KDa (R. Chizzonite, et al., 1992, J. Immunol.,. . .

SUMMARY:

BSUM(6)

Using a non-neutralizing **monoclonal antibody** to the **IL-12** receptor, we have now succeeded in isolating a human cDNA that encodes a low affinity (5-10 nM) **IL-12** receptor. This protein belongs to the cytokine receptor superfamily and within that family shows strongest homology to gp130.

SUMMARY:

BSUM(7)

In order for a molecule such as **IL-12** to exert its effect on cells, it is now accepted by those skilled in the art that the molecule must. . .

SUMMARY:

BSUM(8)

Fanslow, . . . injection of allogeneic cells was completely blocked by soluble IL-1R treatment. What types of therapeutic efficacy that administration of soluble **IL-12** receptor is expected to have can also be contemplated therefor by those skilled in the art.

SUMMARY:

BSUM(9)

The . . . of the purified receptor, in soluble form, presents therapeutic possibilities as well, as shown by Fanslow above. Addition of soluble **IL-12** receptor interferes with the effect of the interleukin on the cells, since the molecule cannot bind to the cell membrane. . . freely. Hence, an aspect of the invention is the treatment of pathological conditions caused by excess activity of cells possessing **IL-12** receptors by adding an amount of soluble **IL-12** receptors sufficient to inhibit binding of **IL-12** to the aforementioned cells. This methodology can also be modified, and the soluble receptor can also be used as a screening agent for pharmaceuticals. Briefly, a pharmaceutical which works as an **IL-12** antagonist can do so by blocking the binding of **IL-12** to the **IL-12** receptor. Prior to determining whether a material would be effective in vivo, one may use the purified **IL-12** receptor in connection with a potential pharmaceutical to determine if there is binding. If not, then the pharmaceutical may no. . .

SUMMARY:

BSUM(11)

The present invention is directed towards an isolated cDNA coding for a human low affinity **IL-12** receptor protein or subunit thereof. When expressed in mammalian cells, the cDNA gives rise to substantially homogeneous **IL-12** receptor protein that binds **IL-12** in a specific and saturable manner with an apparent affinity of about 2 to about 10 nM.

DRAWING DESC:

DRWD(2)

FIGS. 1A, 1B and 1C DNA sequence of human **IL-12** receptor cDNA clone No. 5. (translated portion=nucleotides 65 to 2050) (SEQ ID NO:1).

DRAWING DESC:

DRWD(3)

FIG. 2: Amino acid sequence of human **IL-12** receptor protein as deduced from cDNA sequence of FIGS. 1A, 1B and 1C. (underlined amino acid residues of N-terminal sequence=signal. . .

DRAWING DESC:

DRWD(4)

FIGS. 3A and 3B Alignment of the **IL-12** receptor protein subunit sequence with human gp130, human granulocyte colony-stimulating factor-receptor (G-CSF-R) and leukemia inhibitory factor-receptor (LIF-R), and resulting consensus sequence. Consensus residues indicated by lowercase letters refer to identities between **IL-12** receptor and gp130 only. The following sequence ranges were used: **IL-12** receptor protein (SEQ ID NO:2) residues 42-662 (SEQ. ID NO:4); gp130: residues 124-742 (SEQ. ID NO:5) (M. Hibi et al.,. . .

DRAWING DESC:

DRWD(5)

FIG. 4A and 4B: Scatchard analysis of **IL-12** binding to recombinant human **IL-12** receptor expressed in COS cells.

DRAWING DESC:

DRWD(6)

FIG. 4C and 4D: Scatchard analysis of 2-4E6 antibody binding to recombinant human **IL-12** receptor expressed in COS cells.

DRAWING DESC:

DRWD(7)

FIG. 5: Analysis of the size of human recombinant **IL-12** receptor expressed in COS cells. COS cells transfected with human **IL-12** receptor cDNA were labeled and lysed as described herein. Human **IL-12** receptor protein was immunoprecipitated and the products were analyzed on a 4-20% gradient gel under reducing conditions. 5 .mu.g of each listed antibody were used. They were Control I.sub.g G3=isotype-matched negative control antibody; 2-4E6=anti-human **IL-12** receptor antibody: 4D6 =anti human **IL-12** negative control antibody. Sizes of marker proteins are indicated in KDa on left.

DRAWING DESC:

DRWD(9)

FIG. 7: Inhibition of .sup.125 1-**IL-12** Binding to **IL-12** (IL-12R) Receptor by Mouse Anti-IL-12R Antiserum

DRAWING DESC:

DRWD(10)

FIG. 8: Characterization of: the **IL-12** Binding Proteins on IL-12R Positive Human cells by Affinity-Crosslinking

DRAWING DESC:

DRWD(11)

FIG. 9: Immunoprecipitation of the Solubilized .sup.125 I-**IL-12**/IL-12R Crosslinked Complex by Anti-IL-12R Antibodies

DRAWING DESC:

DRWD(14)

FIG. 12: Inhibition of .sup.125 I-2-4E Binding to K6 Cells by Purified 2-4E6 (24E6), Human **IL-12** (HUIL-12) and Control Antibody (Control IgG)

DRAWING DESC:

DRWD(15)

FIGS. 13A and 13B: Equilibrium Binding of .sup.125 I-**IL-12** to Human K6 Cells at Room Temperature

DRAWING DESC:

DRWD(16)

FIGS. 14A and 14B: Equilibrium Binding of .sup.125 I-**IL-12** to Detergent Solubilized IL-12R from K6 Cells

DRAWING DESC:

DRWD(18)

FIGS. 16A, 16B and 16C: Detection of **IL-12** Receptor on Human Cells by Flow Cytometry

DRAWING DESC:

DRWD(19)

FIG. 17: Size of the **IL-12** receptor subunit on the surface of transfected COS and CTLL cells. 8% gels were used and marker sizes in KDa are indicated. Lanes 1-4: Analysis of affinity crosslinked complexes under non-reducing conditions. Arrowhead=labeled, uncrosslinked 2-4E6 antibody. Arrows=labeled, uncrosslinked **IL-12**. Lanes 5-12: Analysis of .sup.125 I-COS Cell Surface Proteins. Sample reduction, binding of 25 nM unlabeled **IL-12** to the cells prior to analysis and use of 1 mM DTSSP crosslinker are indicated below the lanes. Lanes 13-16:. . .

DRAWING DESC:

DRWD(20)

FIGS. 18A, 18B, 18C, and 18D: Specific inhibition of **IL-12**-induced lymphoblast proliferation by a rat anti-IL-12R antiserum. (18A) Titration by flow cytometry of anti-COS cell antibodies in an anti-IL-12R antiserum. . . . transfected with the human type II IL-1R (--.box-solid.--). (18B-18D) Effects of rat sera on proliferation of PHA-activated PBMC induced by **IL-12** (18B), IL-2 (18C), or IL-7 (18D). All standard errors were <10% of the means.

DRAWING DESC:

DRWD(21)

FIGS. 19A and 19B: Equilibrium binding of .sup.125 I-**IL-12** to COS cells expressing the **IL-12** receptor subunit. 19A) human **IL-12** and 19B) murine **IL-12**. The insets show analysis of

the binding data according to the method of Scatchard.

DETDESC:

DETD(2)

The present invention is directed towards an isolated cDNA (SEQ ID NO:1) that encodes a human low-affinity **IL-12** receptor protein (SEQ ID NO:2) (clone no. 5) or subunit thereof. The amino acid sequence of the substantially homogeneous **IL-12** receptor protein as deduced from the cDNA sequence is shown in FIG. 2 (SEQ ID NO:2). Clone number 17 (SEQ ID NO:3) is also of a substantially homogeneous **IL-12** receptor.

DETDESC:

DETD(3)

The **IL-12** receptor cDNA is useful for the following purposes:

DETDESC:

DETD(4)

Expression of recombinant **IL-12** receptor protein in high levels and its use as an antigen allows production of additional neutralizing monoclonal and polyclonal antibodies. Such neutralizing antibodies can be used in in vivo model settings to elucidate the role that **IL-12** and its receptor play in normal as well as pathologic immune responses (i.e. disease states that are aggravated by activated. . .

DETDESC:

DETD(5)

IL-12 receptor proteins can be administered, for example, for the purpose of suppressing immune responses in a human. A variety of. . . or conditions are caused by an immune response to alloantigen, including allograft rejection and graft-versus-host reaction. In alloantigen-induced immune responses, **IL-12** receptor may suppress lymphoproliferation and inflammation which result upon activation of T cells. **IL-12** receptor may therefore be used to effectively suppress alloantigen-induced immune responses in the clinical treatment of, for example, rejection of. . .

DETDESC:

DETD(6)

IL-12 receptor may also be used in clinical treatment of autoimmune dysfunctions, such a rheumatoid arthritis, diabetes and multiple sclerosis, which are dependent upon the activation of T cells against antigens not recognized as being indigenous to the host. **IL-12** receptor may also be useful in treatment of septic shock in which interferon gamma produced in response to **IL-12** plays a central role in causing morbidity and mortality (G. M. Doherty et al., 1992, J. Immunol. 149:1666).

DETDESC:

DETD(7)

Purified **IL-12** receptor compositions will be useful in diagnostic assays for **IL-12** or **IL-12** receptor, and also in raising antibodies to **IL-12** receptor for use in diagnosis or therapy. In addition, purified **IL-12** receptor compositions may be

used directly in therapy to bind or scavenge **IL-12**, thereby providing a means for regulating the immune or inflammatory activities of **IL-12**. In its use to prevent or reverse pathologic immune responses, soluble **IL-12** receptor can be combined with other cytokine antagonists such as antibodies to the IL-2 receptor, soluble TNF (tumor necrosis factor). . .

DETDESC:

DETD(8)

The dose ranges for the administration of the **IL-12** receptor proteins and fragments thereof may be determined by those of ordinary skill in the art without undue experimentation. In. . . appropriate dosages are those which are large enough to produce the desired effect, for example, blocking the binding of endogenous **IL-12** to its natural receptor. The dosage should not be so large as to cause adverse side effects, such as unwanted. . . the patient, counter indications, if any, immune tolerance and other such variables, to be adjusted by the individual physician. The **IL-12** receptor proteins and fragments thereof can be administered parenterally by injection or by gradual perfusion over time. They can be. . .

DETDESC:

DETD(14)

Recombinant human **IL-12** (U. Gubler et al., 1991, Proc. Natl. Acad. Sci.(U.S.A.), 88:4143) and murine **IL-12** (D. Schoenhaut et al., 1992, J. Immunology, 148:3433) were obtained as described therein.

DETDESC:

DETD(15)

The murine anti human **IL-12** receptor **monoclonal antibody** 2-4E6 used herein was generated as described herein below in Examples 1 to 16 and was purified from ascites fluids. . . by a modification of the lodogen method as described (Pierce Chemical Co., Rockford, Ill.). Radiospecific activities of 5000-7000 cpm/fmole for **IL-12** and 1500-2500 cpm/fmole for the 2-4E6 antibody were typically obtained.

DETDESC:

DETD(17)

The murine anti human **IL-12** receptor **monoclonal antibody** 2-4E6 was prepared, characterized, and generated as set forth in U.S. patent application Ser. No. 08/094,649, filed Jul. 19, 1993,. . .

DETDESC:

DETD(20)

Balb/c . . . supernatant (Nordan, R. P., et al., J. Immunol., 139:813 (1987)) and 100 Units/ml rHuIL-6. Hybridoma supernatants were assayed for specific anti-**IL-12** receptor antibodies by: 1) immunoprecipitation of the soluble complex of .sup.125 I-HuIL-12 crosslinked to **IL-12** receptor (.sup.125 I-**IL-12**/IL-12R); 2) inhibition of .sup.125 I-HuIL-12 binding to PHA-activated PBMCs; and 3) differential binding to **IL-12** receptor positive cells versus receptor negative cells. Hybridoma cell lines secreting specific anti-receptor antibodies were cloned by limiting dilution. Antibodies. . .

DETDESC:

DETD(22)
Preparation of Human PHA Lymphoblasts and **IL-12** Receptor Binding Assays

DETDESC:

DETD(24)

PHA-activated . . . in binding buffer to a cell density of 7.times.10.sup.6 cells/ml. Lymphoblasts (7.times.10.sup.5 cells) were incubated with various concentrations of .sup.125 I-**IL-12** (5-10000 pM) at room temperature for the designated times. Cell bound radioactivity was separated from free .sup.125 -**IL-12** by centrifugation of the assay mixture through 0.1 ml of an oil mixture (1:2 mixture of Thomas Silicone Fluid 6428-R15. . . and cell bound radioactivity was determined in a gamma counter. Non-specific binding was determined by inclusion of 100 nM unlabeled **IL-12** in the assay. Incubations were carried out in duplicate or triplicate. Receptor binding data were analyzed by using the non-linear. . .

DETDESC:

DETD(26)
Affinity Cross-Linking of .sup.125 I-**IL-12** to **IL-12** Receptor Bearing Cell Lines

DETDESC:

DETD(27)

IL-12 receptor bearing cells were incubated with .sup.125 I-**IL-12** (100-500 pM) in the presence or absence of excess unlabeled **IL-12** for 2 hr at room temperature. The cells were then washed with ice-cold PBS pH 8.3 (25 mM Sodium Phosphate. . .

DETDESC:

DETD(29)
Immunoprecipitation Assay of the Soluble Complex of .sup.125 I-**IL-12** Crosslinked to Human IL-12R.

DETDESC:

DETD(30)

For . . . for 2 hr at room temperature. The beads were pelleted by centrifugation, resuspended in 1 ml IP buffer containing .sup.125 I-**IL-12**/IL-12R (10-20,000 cpm) and the mixture was incubated on a rotating mixer for 16 hr at 4.degree. C. After this incubation,. . .

DETDESC:

DETD(32)
Assays for IL-12R Solubilized from Cells Expressing **IL-12** Receptor

DETDESC:

DETD(33)

To . . . and the bound radioactivity determined in a gamma counter. Nonspecific binding was determined by inclusion of 70 nM unlabeled human **IL-12** in the assay. Solubilized IL-12R binding data were analyzed

according to the method of Scatchard using the nonlinear regression programs. . .

DETDESC:

DETD(35)
Competitive Inhibition of .sup.125 I-**IL-12** Receptor Binding by Antibodies

DETDESC:

DETD(36)

The ability of hybridoma supernatant solutions, purified IgG, or antisera to inhibit the binding of .sup.125 I-**IL-12** to PHA-activated lymphoblasts was measured as follows: serial dilutions of culture supernatants, purified IgG or antisera were mixed with activated. . . to each tube and incubated for 1-2 hours at room temperature. Non-specific binding was determined by inclusion of 10 nM unlabeled-**IL-12** in the assay. Incubations were carried out in duplicate or triplicate. Cell bound radioactivity was separated from free .sup.125 I-**IL-12** by centrifugation of the assay through 0.1 ml of an oil mixture as described above. The tip containing the cell. . .

DETDESC:

DETD(38)
Labeling of Human **IL-12** and mAb 2-4E6 with .sup.125 1

DETDESC:

DETD(39)

Human **IL-12** and purified 2-4E6 IgG were labeled with .sup.125 I by a modification of the lodogen method (Pierce Chemical Co., Rockford,. . . 4 min at room temperature. The activated .sup.125 I solution was transferred to a tube containing 0.05 to 0.1 ml **IL-12** (7 .mu.g) or IgG (100 .mu.g) in Tris-iodination buffer and the reaction was incubated for 9 min at room temperature.. . . total radioactivity. The radiospecific activity was typically .about.1500 to 2500 cmp/fmol for 2-4E6 IgG and 5000 to 7000 cpm/fmole for **IL-12**.

DETDESC:

DETD(45)

COS . . . scraping, washed and resuspended in binding buffer. Transfected cells (8.times.10.sup.4) were incubated with increasing concentrations of .sup.125 I-labeled 2-4E6 or **IL-12** at room temperature for 2 hrs. Cell bound radioactivity was separated from free .sup.125 I-labeled 2-4E6 or **IL-12** as described above.

DETDESC:

DETD(50)
Analysis of **IL-12** Receptor Expression on Human Cells by Fluorescence Activated Cell Sorting with mAb 2-4E6

DETDESC:

DETD(51)

To stain cells expressing **IL-12** receptor, 1.times.10.sup.6 cells in 100 .mu.l staining buffer (PBS containing 2% FBS and 0.1% NaN.sub.3) were incubated with 10 .mu.l. . .

DETDESC:

DETD(53)
Inhibition of **IL-12** Binding to Human PHA-Lymphoblasts by Mouse Anti-IL-12R Antiserum.

DETDESC:

DETD(54)

Mice immunized with PHA-activated PBMCs developed an immune response against the human IL-12R as determined by inhibition of .sup.125 I-**IL-12** binding to PHA-activated PBMCs (FIG. 7) and immunoprecipitation of the complex of .sup.125 I-**IL-12** crosslinked to IL-12R (data not shown). The dilutions for half-maximal inhibition of .sup.125 I-**IL-12** binding to PHA-activated.,PBMCs were 1/500 and 1/250 for animals 211-1 and 211-2, respectively (FIG. 7). These antisera also neutralized **IL-12** biologic activity as measured in a PHA-lymphoblast proliferation assay (data not shown). Spleen cells isolated from these mice were fused. . . with SP2/0 myeloma cells and the resulting hybridomas were initially screened for IL-12R specific antibodies by immunoprecipitation of the .sup.125 I-**IL-12**/IL-12R complex and by inhibition of .sup.125 I-**IL-12** binding to IL-12R.

DETDESC:

DETD(55)

For . . . and normal mouse serum (NMS) were preincubated with PHA-activated PBMC for-60 min at RT (room temperature) before addition of .sup.125 I-**IL-12** (100 pM). After addition of .sup.125 I-**IL-12**, the reaction was incubated for 1-2 hrs at RT and the cell bound radioactivity was determined. The data are expressed as the % Inhibition of .sup.125 I-**IL-12** binding in the presence of the immune serum when compared to the specific binding in the absence of serum.

DETDESC:

DETD(58)

The . . . ability of the putative anti-IL-12R antibodies that are immobilized on a solid phase to capture the solubilized complex of .sup.125 I-**IL-12**/IL-12R. To verify that the radioactivity immunoprecipitated by the immobilized antibody was present in the complex of .sup.125 I-**IL-12**/IL-12R, the immunoprecipitated proteins were solubilized, separated by SDS-PAGE and visualized by autoradiography. The preparations of the .sup.125 I-**IL -12**/IL-12R complexes solubilized from PHA-activated PBMC, Kit-225 and K6 cells were resolved into two major radioactive bands, 210-250 KDa and 75 KDa (FIG. 8). The 210-250 KDa and 75 KDa complexes were identified as the .sup.125 I-**IL-12**/IL-12R complex and .sup.125 I-**IL-12** not complexed with the receptor, respectively (FIG. 8). See also R. Chizzonite et al., J. Immunol. 148:3117 (1992). The radioactive 75 KDa band visualized from the cell extracts co-migrated with .sup.125 I-**IL-12**, indicating that it represented .sup.125 I-**IL-12** that bound but was not covalently crosslinked to IL-12R. The 210-250 KDa band was not a covalent crosslinked oligomer of .sup.125 I-**IL-12** because it is not produced when the crosslinking agent BS3 was added directly to .sup.125 I-**IL-12** (FIG. 8).

DETDESC:

DETD(60)

For FIG. 8, PHA-activated PBMC (PHA-PBMC), Kit-225 (Kit-225) and K6 (K6) cells (1.times.10.sup.7 cells/ml) were incubated with .sup.125 I-**IL-12** (100-500 pM) for 2 hrs at room temperature in the absence or presence of 25 nM unlabeled **IL-12**. Cells were then washed, affinity crosslinked with BS3 (0.4 mM final concentration) and a cell extract prepared as described. The. . . by treatment with sample buffer and analyzed by SDS-PAGE and autoradiography on a 8.0% slab gel. The complex of .sup.125 I-**IL-12** crosslinked to the **IL-12** receptor migrates as a single major band of approximately 210-250 KDa. The band migrating at 75 KDa is .sup.125 I-**IL-12** that was bound but not crosslinked to the **IL-12** receptor. .sup.125 I-**IL-12** (**IL-12**) and .sup.125 I-**IL-12** that was treated with the BS3 crosslinker (**IL-12**/BS3) were electrophoresed in parallel lanes as markers for the migration of the 75 KDa **IL-12** heterodimer and for any oligomers of **IL-12** that may form with the BS3 crosslinker. The molecular sizes indicated in the margins were estimated from standards run in. . .

DETDESC:

DETD(61)

TABLE 1

INITIAL IDENTIFICATION OF HYBRIDOMAS SECRETING ANTI-**IL-12** RECEPTOR ANTIBODIES: SPLENOCYTES FROM MICE #211-1 AND #211-2		
HYBRIDOMA/ANTIBODY	I.P. ASSAY.sup.1 (cpm bound)	INHIBITION ASSAY.sup.2

. . .

DETDESC:

DETD(64)

mAb 2-4E6 immunoprecipitates the .sup.125 I-**IL-12**/IL-12R complex solubilized from PHA-activated human lymphoblasts, Kit-225 and K6 cells (FIG. 9, data shown for PHA-activated PBMC), but does not block .sup.125 I-**IL-12** binding to IL-12R expressed on these cells. These data suggested that the 2-4E6 antibody was a non-inhibitory or non-neutralizing anti-IL-12R. . . . between 1500 and 5000 binding sites per cell (PHA-activated PBMC, FIGS. 10A AND 10B; K6 cells, FIGS. 11A and 11B). **IL-12** does not block .sup.125 I-2-4E6 from binding to PHA-activated PBMCs and confirms that 2-4E6 is a non-inhibitory/non-neutralizing antibody (FIG. 12).. . .

DETDESC:

DETD(65)

Equilibrium binding assays have demonstrated that .sup.125 I-**IL-12** identifies 3 separate binding sites on the surface of PHA-activated PBMCs, Kit-225 and K6 cells (FIG. 13, data for K6. . . of Scatchard shows these affinities are approximately 5-20 pM, 50-200 pM and 2-6 nM, respectively. The total number of .sup.125 I-**IL-12** binding sites per cell are approximately 1500 to 5000, which is in good agreement with the total number of binding. . . .sup.125 I-2-4E6 (Table 2). The data also suggests that 2-4E6 recognizes the low affinity (2-5 nM) binding component of the **IL-12** receptor in much the same manner that the anti-TAC antibody recognizes the low affinity component

(p55 subunit) of the IL-2. . . .

DETDESC:

DETD(66)

Since the data indicated that mAb 2-4E6 was a non-neutralizing antibody specific for the IL-12R, the molecular weight and .sup.125 I-**IL-12** binding characteristics of the protein(s) immunoprecipitated by mAb 2-4E6 from the surface of IL-12R positive cells was investigated. The steady state binding of .sup.125 I-**IL-12** to proteins immunoprecipitated by immobilized 2-4E6 from solubilized extracts of PHA-activated PBMCs, Kit-225 and K6 cells was saturable and specific. . . . blot, .sup.125 I-2-4E6 binds to an approximately 90 KDa protein, that is only immunoprecipitated by 2-4E6 and not by an anti-**IL-12** antibody or a control antibody (FIG. 15, data shown for PHA-activated PBMCs). In summary, all the data demonstrated that mAb 2-4E6 bound a protein on the surface of IL-12R positive cells that was approximately 90 KDa and bound .sup.125 I-**IL-12** in a specific manner.

DETDESC:

DETD(67)

For FIG. 9, soluble complexes of .sup.125 I-**IL-12**/IL-12R were prepared from PHA-activiated human PBMC as detailed herein (see also FIG. 8) and immunoprecipitated by immobilized antibodies, 2-4E6, 2C6,. . . . The precipitated proteins were analyzed as described herein and in FIG. 8. Antibodies 4D6 and 20C2 are non-neutralizing and neutralizing anti-**IL-12** antibodies, respectively. 4D6 immunoprecipitates .sup.125 I-**IL-12**/IL-12R complex and free .sup.125 I-**IL-12**, whereas 20C2 only immunoprecipitates free .sup.125 I-**IL-12**. Both 2-4E6 and 2C6 recognize the .sup.125 I-**IL-12**/IL-12R complex. .sup.125 I-**IL-12** (**IL-12**) and .sup.125 I-**IL-12** that was treated with the BS3 crosslinked (**IL-12**/BS3) were electrophoresed in parallel lanes as markers for the migration of the 75 KDa **IL-12** heterodimer and for any oligomers of **IL-12** that may form with the BS3 crosslinker. The molecular sizes indicated in the margins were estimated from standards run in. . .

DETDESC:

DETD(70)

For . . . of 125I-2-4E6 bound [CPM BOUND (Percent)] to the cells in the presence of the indicated concentrations of unlabeled antibody or **IL-12** when compared with the total specific binding in the absence of unlabeled competitor.

DETDESC:

DETD(71)

For . . . 13A and 13B, K6 cells (1.times.10.sup.6 cells) were incubated for 2 hrs at room temperature with increasing concentrations of .sup.125 I-**IL-12** in the absence (.smallcircle.) or presence (.circle-solid.) of 50 nM unlabeled **IL-12**. Total (.smallcircle.) and non-specific (.circle-solid.) cell bound radioactivity were determined as described. Specific binding of .sup.125 I-**IL-12** (.gradient.) was calculated by subtracting non-specific binding from total binding. The right hand panel shows analysis of the binding data. . .

DETDESC:

DETD(72)

For . . . coupled to agarose as described. Following this incubation, the beads were pelleted, washed and resuspended in IP buffer containing .sup.125 I-**IL-12** at concentrations ranging from 7 pM to 7.5 nM. The IL-12R immobilized on the 2-4E6 coated beads was incubated with .sup.125 I-**IL-12** for 2 hrs at RT and IL-12R bound radioactivity was determined in the presence of 50 nM unlabeled **IL-12**. The right hand panels show analysis of the binding data according to the method of Scatchard as determined by LIGAND. . . .

DETDESC:

DETD(74)

TABLE 2

COMPARISON OF THE BINDING OF **IL-12** AND 2-4C6 TO HUMAN CELLS EXPRESSING **IL-12** RECEPTOR

	IL-12 BINDING.sup.1		2-4E6 BINDING.sup.2	
CELL TYPE	K.sub.D (nM)	Sites/cell	K.sub.D (nM)	Sites/cell
Human Cells				
non-activated				
	none	detected. . . .		

DETDESC:

DETD(77)

The . . . cDNA was engineered in a mammalian cell expression vector, transfected into COS-7 cells and the specificity for binding of .sup.125 I-**IL-12** and .sup.125 I-2-4E6 was determined. Steady state binding of .sup.125 I-**IL-12** to the rIL-12R expressing COS cells identifies a single binding site with an apparent affinity of 2-6 nM and approximately 150,000 sites/cell (FIGS. 4A and 4B). This low affinity **IL-12** binding site corresponds to the low affinity site seen in the binding assays with human cells that naturally express IL-12R.. . . . identifies approximately 500,000 sites/cell (FIGS. 4C and 4D). COS cells transfected with an unrelated plasmid do not bind either .sup.125 I-**IL-12** or .sup.125 I-2-4E6 (data not shown). These data demonstrated unequivocally that mAb 2-4E6 was specific for the low affinity component. . . .

DETDESC:

DETD(78)

For . . . Three days later, transfected cells (1.times.10.sup.4 cells) were incubated for 2 hrs at room temperature with increasing concentration of .sup.125 I-**IL-12** in the absence (.smallcircle.) or presence (.quadrature.) of 50 nM unlabeled **IL-12**. Total (.smallcircle.) and non-specific (.quadrature.) cell bound radioactivity were determined as described. Specific binding of .sup.125 I-**IL-12** (.tangle-solidup.) was calculated by subtracting non-specific binding from total binding. The right hand panel shows analysis of the binding data. . . .

DETDESC:

DETD(83)

For . . . obtained in the presence of a control antibody specific for IL-1 receptor (anti-Hu IL-1R), a control antibody specific for human **IL-12** (4D6+GART-PE CTRL) and the goat anti-mouse antibody conjugated with PE (GART-PE CTRL).

DETDESC:

DETD(85)

Peripheral . . . rhuIL-2 (Hoffmann-La Roche Inc.) to yield >95% T-cells. The next day, these cells were used for assessing their responsiveness to **IL-12**, for radioligand (**IL-12**) binding assays and in flow cytometry assays for the detection of **IL-12** receptors.

DETDESC:

DETD(86)

Flow cytometric detection of **IL-12** receptors on such 4 day activated PHA blasts was performed as follows: the cells were washed twice in PBS and. . . azide. All the subsequent steps were carried out at 4 degrees C. 1.times.10.sup.6 cells were incubated in 1 nM human **IL-12** for 40 minutes. The cells were washed in FACS buffer and incubated with about 1 .mu.g of biotinylated rat anti human p40 **IL-12** subunit antibody 4D6 for 20 minutes. Cells were washed again and resuspended in 100 .mu.l of a 5 .mu.g/ml streptavidin-phycoerythritin. . .

DETDESC:

DETD(89)

RNA . . . .mu.g/ml denatured salmon sperm carrier DNA at 37.degree. C. overnight. The probe was generated by random-primer labeling gel-isolated insert from **IL-12** receptor cDNA clone No.5 by the method as described in Molecular Cloning Manual. The blots were first quickly rinsed at. . .

DETDESC:

DETD(92)
Screening for **IL-12** receptor cDNAs by panning

DETDESC:

DETD(93)

The . . . 0.5 mM EDTA/0.02% Na Azide in PBS and a single cell suspension was prepared for each pool. The monoclonal anti **IL-12** receptor antibody 2-4E6 as discussed above was subsequently bound to the cells in suspension (10 .mu.g/ml in PBS-0.5 mM EDTA-0.02%. . .

DETDESC:

DETD(94)

From . . . one-chamber microscopic slides (2 slides per pool). 2-3 days after transfection, to one of the slides was bound labeled human **IL-12** (10.sup.6 cpm/ml=300 pM in RPMI 1640 plus 5% FCS for 2-3 hours at 4 degrees C.) and to the other. . . fold bright field magnification. One of the ten pools, number 5, showed a large number of positive cells both for **IL-12** and 2-4E6 binding. E coli clones from this 3.times. panned pool were subsequently picked into a

microtiterplate (3 clones per. . . into COS cells on 12 well plates (10.sup.5 cells well, 4 wells per pool). 2-3 days after the transfection, labeled **IL-12** was bound to the cells in two wells (total binding), whereas the other two wells per pool received labeled **IL-12** and a 100 fold molar excess of cold **IL-12** (=nonspecific binding). Wells were washed and the bound radioactivity eluted with 0.5 ml of 1% SDS and counted in a. . . and the other one representing row F from the microtiterplate E. coli clones from well F1 must thus contain the **IL-12** binding activity. Clones from that well were plated, and DNA from 10 single colonies was analyzed for plasmid insert size.. . . 3 out of the 10 colonies showed an insert of about 2.1 kilobases in length, large enough to encode the **IL-12** receptor. One of these- clones was picked for further analysis.

DETDESC:

DETD(95)
Characterization of **IL-12** receptor cDNAs

DETDESC:

DETD(96)

IL-12 receptor clone No. 5 was picked as described above and the plasmid DNA isolated. Gel isolated insert was sequenced on. . .

DETDESC:

DETD(98)

Cloned **IL-12** receptor cDNAs were expressed in COS cells using either the DEAE dextran transfection or electroporation techniques (Molecular Cloning Manual). Binding assays with labeled **IL-12** or labeled 2-4E6 antibody were run as described hereinabove under anti human **IL-12** receptor antibody. The binding data were analyzed and Kd values were calculated according to Scatchard, using the LIGAND program discussed hereinabove under anti human **IL-12** receptor antibody. In vivo labeling (6 hours) of COS cells (3.times.10.sup.5 cells per 35 mm diameter tissue culture dish) with. . . ml, Genex) and normal mouse serum (25% final concentration) at 4.degree. C. overnight. The beads were spun out and labeled **IL-12** receptor was specifically immunoprecipitated from the cleared lysates by adding 5 .mu.g of 2-4E6 antibody per ml of sample. The. . .

DETDESC:

DETD(100)

Lymphocyte . . . sera were mixed in the wells of a 96-well plate and incubated at 37.degree. C. for 30 min. The cytokines (**IL-12**, IL-2 or IL-7) were then added to the wells, and the cultures were incubated for 48 h at 37.degree. C.. . .

DETDESC:

DETD(103)
Inhibition of **IL-12**-induced lymphoblast proliferation by anti-IL-12R anti serum

DETDESC:

DETD(104)

To determine whether the cloned IL-12R subunit plays an essential role in an **IL-12**-induced biologic response, we examined whether

antiserum to the. cloned IL-12R subunit could inhibit IL-12induced proliferation of PHA-activated PBMC. This antiserum. . . antiserum, the anti-IL-1R antiserum, and preimmune serum (from the rat used to prepare the anti-IL-12R) on lymphoblast proliferation induced by **IL-12**, IL-2, or IL-7. The concentrations of **IL-12**, IL-2, and IL-7 were 0.25 ng/ml, 1.25 ng/ml, and 0.4 ng/ml, respectively. These concentrations were chosen because they resulted in. . . levels of .sup.3 H-TdR incorporation in the presence of saturating amounts of cytokine were 38,820, 111,303, and 89,541 cpm for **IL-12**, IL-2, and IL-7, respectively. The level of .sup.3 H-TdR incorporation in the absence of any added cytokine is indicated by. . . with essentially identical results, and one of these is illustrated in FIGS. 18B-D. The anti-IL-12R antiserum caused dose-dependent inhibition of **IL-12**-induced lymphoblast proliferation but had no effect on proliferation induced by IL-2 or IL-7. In contrast, neither the preimmune serum nor. . .

DETDESC:

DETD(105)
Sequence Analysis of **IL-12** receptor cDNA clones

DETDESC:

DETD(106)

The DNA sequence for the **IL-12** receptor cDNA insert from clone No. 5 is shown in FIGS. 1A, 1B and 1C (SEQ ID NO:1). The deduced amino acid sequence for the encoded receptor-protein is shown in FIG. 2. The **IL-12** receptor protein is thus composed of 662 amino acids and a calculated molecular weight of 73,112. The **IL-12** receptor protein has the following features: N-terminal signal peptide, extracellular domain, transmembrane domain and cytoplasmic tail. The classical hydrophobic N-terminal. . . after the amino acids Ala, Ser, Gly, Cys, Thr, Gin (G. von Heijne, Nucl. Acids Research, 1986, 14:4683). For the **IL-12** receptor, the cleavage could thus take place after Gln20, Ala23 or Cys24 in the sequence shown in FIG. 2, leaving. . .

DETDESC:

DETD(107)

The . . . additional 202 bp of 3' untranslated region. The amino acid sequence (SEQ ID NO:3) deduced from this clone for the **IL-12** receptor protein was almost completely identical to the sequence shown in FIG. 2 (SEQ ID NO:2) ; however, a 13. . .

DETDESC:

DETD(108)

Further analysis of the amino acid sequence of the **IL-12** receptor shows it to be a member of the cytokine receptor superfamily, by virtue of the sequence motifs [Cys52 - - - Cys62SW] and [W222SKWS]. Comparing the **IL-12** receptor sequence to all the members of the superfamily by running the ALIGN program shows that the **IL-12** receptor has the highest homology to human gp130.

DETDESC:

DETD(109)

Sequence analysis of the **IL-12** receptor extracellular domain demonstrated the presence of the hemopoietin receptor hallmark features: two pairs of conserved cysteine-residues and the WSXWS motif Further

comparisons to the hemopoietin receptor superfamily showed the newly isolated. **IL-12** receptor component (SEQ: ID NO:5) to be highly related to a subgroup of family members composed of gp130 (SEQ: ID. . . . (SEQ: ID NO:7) (FIGS. 3A and 3B); align scores were 12.37 (IL-12R/gp130), 7.35 (IL-12R/G-CSF-R) and 6.31 (IL-12R/LIF-Rbeta). Similarities between the **IL-12** receptor component and these three proteins extend beyond the hemopoietin receptor domain and include the area from the WSXWS motif. . . . (A. R. Kornblihtt, et al., 1985, EMBO J., 4:1755; L. Patthy, 1990, Cell, 61:13). Similarly, the extracellular domain of the **IL-12** receptor can be subdivided into five such fibronectin type III repeats (residues 43-133, 143-236, 237-337, 338-444 and 445-540). The **IL-12** receptor extracellular domain lacks the N-terminal Ig domain found in gp130 and therefore only accommodates 5 fibronectin type III repeats. Further sequence similarities between the **IL-12** receptor, gpl130 (SEQ: ID NO:5), the G-CSF-receptor (SEQ: ID NO:6) and the LIF-receptor (SEQ: ID NO:7) can be found in. . . . Nati. Acad. Sci. (U.S.A.), 88:11349). Both·those motifs are also found in conserved areas of the cytoplasmic part of the **IL-12** receptor sequence (amino acid residues 577-584 and amino acid residues 618-629).

DETDESC:

DETD(110)
Analysis of **IL-12** Receptor mRNA Expression

DETDESC:

DETD(111)

RNA blots were performed using poly A+ RNA from cells known to respond to **IL-12**: PHA-stimulated PBMC and the CD4+ T-cell line Kit225. Two RNA transcripts about 3 Kb and 2.3 Kb in size are. . . . derived from the cytoplasmic domain (lanes 4-6). This finding could indicate the presence of an RNA encoding i) a soluble **IL-12** receptor protein, ii) a membrane bound **IL-12** receptor devoid of a cytoplasmic region altogether or iii) an **IL-12** receptor with a cytoplasmic sequence that is completely different from the ones present in clones 5 and 17. Elucidation of. . . .

DETDESC:

DETD(112)
Characterization of Recombinant **IL-12** Receptor

DETDESC:

DETD(113)

IL-12 receptor cDNA (clone number 5) (SEQ ID NO:1) was electroporated into COS cells and equilibrium binding of labeled human and murine **IL-12** to the cells was performed and analyzed as described (R. Chizzonite, et al., 1992, J. Immunol., 148:3117). Results are shown in FIGS. 19A and 19B. humane and murine **IL-12** bind to recombinant **IL-12** receptor (SEQ ID NO:2) with a single affinity (K.sub.D) of 3.4.+-.1.3 nM (n=7) and 2.1.+-.0.5 nM (n=4), respectively, which corresponds to the low affinity component of the functional **IL-12** receptor on PHA-activated PBMC. After transformation by the method of Scatchard, the equilibrium binding data was best described by a. . . . by the LIGAND program. The site numbers indicated in FIG. 19 are calculated assuming that all cells are expressing receptors. **IL-12** receptor protein expressed by clone number 17 (SEQ ID NO:3) gave similar results in these binding assays. SEQ ID NO:3. . . .

DETDESC:

DETD(114)

Metabolic labeling and immunoprecipitation of the **IL-12** receptor subunit expressed in COS cells indicated its size to be about 100 KDa as determined by gel analysis under. . . the size of the receptor at the cell surface, affinity crosslinking studies were performed. Unless otherwise stated, characterization of the **IL-12** receptor protein was done on SEQ ID NO:2. Crosslinking of 0.2 nM .sup.125 I-labeled **IL-12** to either transfected COS cells, PHA-activated PBMC or K6 cells gave rise to complexes that migrate at >200 KDa (FIG. 17, lanes 1,3 and 4; arrow indicates uncrosslinked **IL-12**). Crosslinking at 2 nM .sup.125 I-**IL-12** (a concentration equivalent to the K.sub.D) gave identical results (not shown). The size of a complex composed of one receptor subunit and one **IL-12** ligand is expected to be about 175 KDa. However, FIG. 17 shows that the 175 KDa complex is present only. . . the 150 kDA Ig and the 200 KDa markers are not separated on the gel system used, the 175 KDa **IL-12/IL-12** receptor complex is expected to comigrate with them. For comparison, lane 2 shows transfected COS cells crosslinked to labeled 2-4E6 antibody (arrowhead=uncrosslinked 2-4E6). Crosslinking labeled **IL-12** to i) cells that do not bind **IL-12** (e.g. Raji cells), ii) mock-transfected COS cells or iii) transfected COS cells in the presence of an excess of cold **IL-12** did not yield any products (not shown). For FIG. 17, labeled **IL-12** (0.2 nM) was bound and crosslinked with BS3 (0.4 mM) to transfected COS cells (lane 1), PHA-activated PBMC (lane 3). . . 4). Labeled 2-4E6 antibody (0.5 nM) was bound and crosslinked with BS3 (0.4 mM) to transfected COS cells (lane 2). Anti-**IL-12** receptor antibody 2-4E6 (lanes 5,7), anti IL12 antibody 4D6 (lanes 9,11) and control antibody (lanes 6,8,10,12,) were used. Antibody 2-4E6. . .

DETDESC:

DETD(115)

Since crosslinking of labeled **IL-12** to **IL-12** receptor gave rise to products that are larger than what is expected for a complex of one receptor and one **IL-12** ligand but whose size is difficult to estimate, cell surface labeling and immunoprecipitation experiments of transfected COS cells were performed.. . . and nonreducing conditions (FIG. 17, lanes 5-12). The results can be summarized as follows: i) transfected COS cells express the **IL-12** receptor subunit both as monomers and as a second, larger product that could be dimers or oligomers. Both these products are present at about depend on **IL-12** binding. If **IL-12** is prebound to the cells, the resulting banding pattern does not change (not shown); and iii) The dimers/oligomers can be. . . 7). The data from the crosslinking and surface labeling experiments thus suggested that only the dimeric/oligomeric receptor subunit form binds **IL-12** with the 3 nM affinity measured on transfected COS cells. This possibility was further investigated as follows. Complexes produced by binding unlabeled **IL-12** to .sup.125 I-surface labeled COS cells and crosslinking with a cleavable crosslinker were immunoprecipitated by an anti-**IL-12** antibody and analyzed under non-reducing and reducing conditions (FIG. 17, lanes 9-12). The anti-**IL-12** antibody only precipitated a complex corresponding to **IL-12** bound to the dimer/oligomer but not the monomer of the **IL-12** receptor subunit (lane 9). Analysis of this complex under reducing conditions identified a labeled protein that co-migrated with the **IL-12** receptor monomer (lane 11). Experiments with a murine CTLL cell transfectant stably expressing the **IL-12** receptor subunit lend further support to our findings. These cells express about 3000 to 5000 receptor subunits per cell, as measured by 2-4E6 antibody binding; however, the cells bind **IL-12** very inefficiently, with an estimated Kd of 50 nM or greater (not shown). The results from surface labeling and immunoprecipitation experiments with the CTLL transfectants clearly

indicate that they only express **IL-12** receptor subunit monomers (FIG. 17, lanes 13-16). Taken together, the data support the hypothesis that only the receptor subunit dimers/oligomers bind **IL-12** with the low affinity (3 nM) measured on transfected COS cells.

DETDESC:

DETD(117)

We . . . 5; SEQ ID NO:1) coding for a type I transmembrane protein that represents a low affinity component of the functional **IL-12** receptor (SEQ ID NO:2) found on PHA-activated PBMC. Several lines of evidence are available to support this claim. i) When transfected into COS cells, the cDNA confers specific **IL-12** binding to the cells. ii) The affinity of this receptor-ligand interaction is about 3 nM, which corresponds to the low affinity **IL-12** receptor component observed on PHA blasts. iii) The recombinant **IL-12** receptor component expressed in COS cells binds both human and murine **IL-12** ligands with comparable affinity. This is expected, since it was shown that similar concentrations of human and murine **IL-12** transduce a signal through the human **IL-12** receptor (D. S. Schoenhaut, et al., 1992, J. Immunol., 148:3433). iv) The 2-4E6 antibody recognizes both the recombinant receptor component expressed in COS cells and a component of the **IL-12** receptor expressed on PHA-activated PBMC and K6 cells. 2-4E6 immunoprecipitates the complex of .sup.125 I-**IL-12** affinity crosslinked to the functional **IL-12** receptor on activated PBMC and K6 cells. v) Polyclonal antiserum from a rat immunized with whole COS cells transfected with the **IL-12** receptor subunit inhibits proliferation of PHA-activated PBMC induced by **IL-12**, but not IL-2 or IL-7 induced proliferation. Whether it also plays an essential role in other **IL-12**-induced responses, such as IFN-gamma production by resting PBMC or NK cell activation, remains to be determined. Dual label flow cytometry has shown that the **IL-12** receptor subunit is upregulated on NK-cells cultured with IL-2, consistent with our previous observations that IL-2 caused upregulation of **IL-12** receptors on NK-cells (B. B. Desai, et al., 1992, J. Immunol., 148:3125). No neutralizing **monoclonal antibodies** to the **IL-12** receptor subunit are currently available.

DETDESC:

DETD(118)

The size of the **IL-12** receptor subunit at the cell surface was estimated by affinity crosslinking of labeled **IL-12** as well as cell-surface labeling studies. Transfected COS cells express the **IL-12** receptor subunit as a protein of about 100 KDa size. The calculated molecular weight for the mature form of the. . . surface expressed protein is likely to be carbohydrate. Transfected COS cells also express a larger molecular weight form of the **IL-12** receptor subunit. Our present working hypothesis, but to which we do not wish to be bound, is that this form. . .

DETDESC:

DETD(119)

The available evidence supports the conclusion that the **IL-12** receptor dimerization/oligomerization is independent of **IL-12** binding. Similar to these findings, it has been reported for the EPO receptor that disulfide-bonded receptor dimers and oligomers are. . . effect on receptor dimerization (O. Miura, et al., 1993, Archives Biochem. Biophys., 306:200). Our data also indicate that only the **IL-12** receptor dimers/oligomers bind **IL-12** with the 2-5 nM affinity observed on intact transfected COS cells. i) An

anti-**IL-12** antibody only immunoprecipitates an affinity crosslinked complex corresponding to one **IL-12** and a receptor dimer/oligomer. ii) Affinity crosslinked complexes of the size expected for one receptor subunit and one **IL-12** are formed very inefficiently at **IL-12** concentrations corresponding to the KD measured on transfected COS cells. iii) Murine CTLL cells stably expressing the receptor subunit bind **IL-12** very inefficiently (estimated KD=50 nM or lower); these cells also do not express subunit dimers/oligomers. It was unexpected to find that COS cells and CTLL cells differ in their ability to express the **IL-12** receptor subunit in a way that allows **IL-12** binding. This could be due to species specificity: murine CTLL cells are somehow unable to "process" the human **IL-12** receptor protein correctly, resulting in inefficient dimerization/oligomerization and **IL-12** binding. Conceivably, COS cells could express an endogenous protein that allows the **IL-12** receptor dimerization/oligomerization to occur. Since under the experimental conditions used, the number of low-affinity **IL-12** receptor sites per transfected COS cell is always greater than 10.sup.5, it seems unlikely that an endogenous COS cell component. . .

DETDESC:

DETD(120)

The **IL-12** receptor subunit that we have isolated is a member of the hemopoietin receptor superfamily. Within that family, it is most. . . closely related over its entire length to gp130 and the receptors for G-CSF and LIF. The extracellular portion of the **IL-12** receptor subunit can also be divided into five fibronectin type III repeats, similar to what was reported for gp130 (M. Hibi, et al., 1990, Cell, 63:1149). It is interesting to note that the ligands for **IL-12** receptor and gp130, i.e. **IL-12** p40 and IL-6 receptor, both also contain such fibronectin type III repeats (M. Hibi, et al., 1990, Cell, 63:1149; D. S. Schoenhaut, et al., 1992, J. Immunol., 148:3433). Some features of the cytoplasmic portion of the **IL-12** receptor subunit merit further comment. Compared to the corresponding areas in gp130 (276 AA) and the receptor for LIF (237. . . to transduce a signal (M. Murakami, et al., 1991, Proc. Natl. Acad. Sci. (U.S.A.), 88:11349). The potential functionality of the **IL-12** receptor cytoplasmic portion appears to be borne out further by the presence of a number of features conserved in other. . . (M. Murakami, et al., 1991, Proc. Natl. Acad. Sci. (U.S.A.), 88:11349) are clearly present and thus give the low affinity **IL-12** receptor component the makeup of a beta type subunit (N. Stahl, et al., 1993, Cell, 74:587).

DETDESC:

DETD(121)

Some reports in the past have drawn analogies between the IL-6 and **IL-12** systems. Because homologies exist between i) the **IL-12** p35 subunit and IL-6 (D. M. Merberg, et al., 1991, Immunology Today, 13:78) and ii) the **IL-12** p40 subunit and the extracellular domain of the IL-6 receptor, **IL-12** has been viewed as a complex between a soluble receptor (p40) and a cytokine (p35) (D. P. Gearing, et al., 1991, Cell, 66:9). It was predicted that the **IL-12** receptor would be homologous to gp130 (D. Cosman, 1993, Cytokine, 5:95). Our results clearly support this prediction; however, they also demonstrate differences between the IL-6/soluble IL-6 receptor/gp130 system and the **IL-12/IL-12** receptor system. Expression of gp130 occurs in a wide variety of cells and in an almost constitutive fashion (T. Taga, et al., 1992, FASEB J., 6:3387); the **IL-12** receptor subunit and its mRNA are highly inducible in PBMC. Gp130 acts as an affinity converter for the IL-6/IL-6 receptor interaction (T. Taga, et al., 1992, FASEB J., 6:3387); the **IL-12**

US PAT NO: **5,536,657** [IMAGE AVAILABLE] L21: 1 of 1
TITLE: Recombinant DNA encoding human receptor for interleukin-12

ABSTRACT:
This invention relates to substantially pure Interleukin-12 receptor cDNAs and protein and uses therefore. The Interleukin-12 receptor is shown to be a member of the cytokine receptor superfamily and has a high homology to human gp130.

DETDESC:

DETD(10)

As . . . DNA isolated at least once in substantially pure form, i.e., free of contaminating endogenous materials and in a quantity or **concentration** enabling identification, manipulation, and recovery of the sequence and its component nucleotide sequences by standard biochemical methods, for example, using. . .

DETDESC:

DETD(20)

Balb/c . . . of Fazekas et al., J. Immunol. Methods 35, 1 (1980). The fused cells were plated at a density of 6.times.10.sup.5 cells/**ml**/well in 48-well cluster dishes in IMDM supplemented with 10% FBS, glutamine (2 mM), b-mercaptoethanol (0.1 mM), gentamicin (50 g/**ml**), 5% ORIGEN hybridoma cloning factor (IGEN, Inc.), 5% P388D1 supernatant (Nordan, R. P., et al., J. Immunol., 139:813 (1987)) and 100 Units/**ml** rHuIL-6. Hybridoma supernatants were assayed for specific anti-IL-12 receptor antibodies by: 1) immunoprecipitation of the soluble complex of .sup.125 I-HuIL-12. . .

DETDESC:

DETD(23)

Human . . . Gately et al., J. Natl. Cancer Inst. 69, 1245 (1982)) and cultured at 37.degree. C. at a density of 5.times.10.sup.5 cells/**ml** in tissue culture medium (TCM) containing 0.1% PHA-P (Difco). After 3 days, the cultures were split 1:1 with fresh TCM, and human rIL-2 was added to each culture to give a final **concentration** of 50 units/**ml**. The cultures were then incubated for an additional 1-2 days prior to use in assays.

DETDESC:

DETD(24)

PHA-activated . . . the designated times. Cell bound radioactivity was separated from free .sup.125 I-IL-12 by centrifugation of the assay mixture through 0.1 **ml** of an oil mixture (1:2 mixture of Thomas Silicone Fluid 6428-R15 {A. H. Thomas} and Silicone Oil AR 200 {Gallard-Schlessinger}). . .

DETDESC:

DETD(27)

IL-12 . . . PBS pH 8.3 (25 mM Sodium Phosphate pH 8.3, 0.15 M NaCl and 1 mM MgCl.sub.2) and resuspended at a **concentration** of 0.5-1.0.times.10.sup.7 cells/**ml** in PBS pH 8.3. BS3 (Pierce) in dimethyl sulfoxide was added to a final **concentration** of 0.4 mM. Incubation was continued for 30 min. at 4.degree. C. with constant agitation. The cells were washed with ice-cold 25 mM Tris-HCl (pH 7.5), 0.15 M NaCl and 5 mM EDTA and then solublized at 0.5-1.0.times.10.sup.8 cells/**ml** in solubilization buffer (50 mM Tris-HCl (pH 8.0) containing 8 mM CHAPS, 0.25 M NaCl, 5 mM EDTA, 40 .mu.g/**ml** PMSF, 0.05% NAN.sub.3, and 1% BSA) for 1 hr at 4.degree. C. The extracts were centrifuged at 12,000.times.g for 45. . .

DETDESC:

DETD(30)

For the immunoprecipitation assay, hybridoma culture supernatant (0.5 **ml**), diluted antisera, or purified IgG was added to a microfuge tube containing 0.1 **ml** of a 50% suspension of either goat-anti-mouse IgG coupled to agarose (Sigma Chem. Co.) or Protein G coupled to Sepharose 4B (Pharmacia). The assay volume was brought up to 1.0 **ml** with IP buffer (8 mM CHAPS in PBS (0.25 M NaCl), 1% BSA, & 5 mM EDTA) and the mixture. . . incubated on a rotating mixer for 2 hr at room temperature. The beads were pelleted by centrifugation, resuspended in 1 **ml** IP buffer containing .sup.125 I-IL-12/IL-12R (10-20,000 cpm) and the mixture was incubated on a rotating mixer for 16 hr at. . .

DETDESC:

DETD(33)

To . . . the assay. See Stern and Podlaski, Techniques in Protein Chemistry (1993). The immobilized antibodies were resuspended in IP buffer (0.3 **ml**) and 0.2 **ml** of a detergent solubilized extract of PHA-activated PBMCs or K6 cells that contained IL-12R was added. To prepare the detergent. . . washed with ice-cold 25 mM Tris-HCl (pH 7.5), 0.15 M NaCl and 5 mM EDTA and then solublized at 1.5.times.10.sup.8 cells/**ml** in solubilization buffer (50 mM Tris-HCl, pH 8.0, containing 8 mM CHAPS, 0.25 M NaCl, 5 mM EDTA, 40 .mu.g/**ml** PMSF, 0.05% NAN.sub.3, and 1% BSA) for 1 hr at 4.degree. C. The extracts were centrifuged at 120,000.times.g for 60. . . 16 hr at 4.degree. C. After this incubation, the beads were pelleted by centrifugation and resuspended in IP buffer (0.15 **ml**) containing .sup.125 I-HuIL-12 at concentrations ranging from 0.05 to 7.5 nM. The IL-12R immobilized on the antibody coated beads was. . .

DETDESC:

DETD(36)

The . . . in duplicate or triplicate. Cell bound radioactivity was separated from free .sup.125 I-IL-12 by centrifugation of the assay through 0.1 **ml** of an oil mixture as described above. The tip containing the cell pellet was excised, and cell bound radioactivity was. . .

DETDESC:

DETD(39)

Human . . . borosilicate glass tube. For radiolabeling, 1.0 mCi Na[.sup.125 I] (Amersham, Chicago, Ill.) was added to an Iodogen-coated tube containing 0.05 **ml** of Tris-iodination buffer (25 mM Tris-HCL pH

7.5, 0.4 M NaCl and 1 mM EDTA) and incubated for 4 min at room temperature. The activated .sup.125 I solution was transferred to a tube containing 0.05 to 0.1 **ml** IL-12 (7 .mu.g) or IgG (100 .mu.g) in Tris-iodination buffer and the reaction was incubated for 9 min at room temperature. At the end of the incubation, 0.05 **ml** of Iodogen stop buffer (10 mg/**ml** tyrosine 10% glycerol in Dulbecco's PBS, pH 7.40) was added and reacted for 3 min. The mixture was then diluted with 1.0 **ml** Tris-iodination buffer, and applied to a Bio-Gel P10DG desalting column (BioRad Laboratories (BRL)) for chromatography. The column was eluted with Tris-iodination buffer, and fractions (1 **ml**) containing the peak amounts of labeled protein were combined and diluted to 1.times.10.sup.8 cpm/**ml** with 1% BSA in Tris-iodination buffer. The TCA preciptable radioactivity (10% TCA final **concentration**) was typically in excess of 95% of the total radioactivity. The radiospecific activity was typically.about.1500 to 2500 cmp/fmol for 2-4E6. . .

DETDESC:

DETD(42)

PHA-activated . . . 1.5 hrs. Cell bound radioactivity was separated from free .sup.125 I-2-4E6 IgG by centrifugation of the assay mixture through 0.1 **ml** silicone oil at 4.degree. C. for 90 seconds at 10,000.times.g. The tip containing the cell pellet was excised, and cell. . .

DETDESC:

DETD(48)

PHA-activated PBMC were washed 3 times with ice-cold PBS and solubilized at 0.5-1.times.10.sup.8 cells/**ml** in solubilization buffer (50 mM TrisHCl pH 8.0 containing 8 mM CHAPS, 0.25 M NaCl, 5 mM EDTA, 40 .mu.g/**ml** PMSF, 0.05% NaN.sub.3 and 1 mg/**ml** BSA)for 1 hr at 4.degree. C. The extracts were centrifuged at 12,000.times.g for 45 min. at 4.degree. C. to remove. . . (5.0% w/v nonfat dry milk in PBS +0.05% Tween 20) and duplicate blots were probed with .sup.125 I-2-4E6 IgG (1.times.10.sup.6 cpm/**ml** in 8 mM CHAPS in PBS, 0.25 M NaCl, 10% BSA and 5 mM EDTA)+unlabeled 2-4E6 IgG (67 nM).

DETDESC:

DETD(60)

For FIG. 8, PHA-activated PBMC (PHA-PBMC), Kit-225 (Kit-225) and K6 (K6) cells (1.times.10.sup.7 cells/**ml**) were incubated with .sup.125 I-IL-12 (100-500 pM) for 2 hrs at room temperature in the absence or presence of 25 nM unlabeled IL-12. Cells were then washed, affinity crosslinked with BS3 (0.4 mM final **concentration**) and a cell extract prepared as described. The cell extract was precipitated with wheat germ lectin bound to solid supports. . .

DETDESC:

DETD(72)

For FIG. 14, K6 cells (1.5.times.10.sup.8 cells/**ml**) were solubilized with 8 mM CHAPS extraction buffer and the cell extract (0.2 **ml**) was immunoprecipitated for 16 hrs at 4.degree. C. with mAb 2-4E6 immobilized on goat anti-mouse IgG coupled to agarose as. . .

DETDESC:

DETD(73)

For FIG. 15, PHA-activated PBMC (1.times.10.sup.8 cells/**ml**) were solubilized with 8 mM CHAPS extraction buffer and the cell extract (1 **ml**) was immunoprecipitated as described in FIG. 14. Following this incubation, the beads were pelleted, washed and the bound proteins released. . .

DETDESC:

DETD(78)

For . . . rIL-12R as described. Three days later, transfected cells (1.times.10.sup.4 cells) were incubated for 2 hrs at room temperature with increasing **concentration** of .sup.125 I-IL-12 in the absence (.largecircle.) or presence (.quadrature.) of 50 nM unlabeled IL-12. Total (.largecircle.) and non-specific (.quadrature.). . .

DETDESC:

DETD(85)

Peripheral . . . at room temperature. PBMC at the interface were collected and pelleted at 1500 rpm for 10 minutes through a 15 **ml** cushion of 20% sucrose in PBS. Pelleted PBMC were resuspended in tissue culture medium and washed twice in the same medium (RPMI 1640 plus 5% serum). Finally, the cells were cultured at 0.5-1.times.10.sup.6 cells/**ml** in tissue culture medium plus 1 .mu.g/**ml** PHA-P (Difco) for 3 days at 37 degrees C. in a 5% CO2 atmosphere. Cells were split 1:1 in culture medium plus 50 U/**ml** rhuIL-2 (Hoffmann-La Roche Inc.) to yield >95% T-cells. The next day, these cells were used for assessing their responsiveness to. . .

DETDESC:

DETD(86)

Flow . . . 4 day activated PHA blasts was performed as follows: the cells were washed twice in PBS and resuspended at 2.times.10.sup.6 cells/**ml** in PBS plus 2% fetal calf serum and 0.1% sodium azide. All the subsequent steps were carried out at 4. . . p40 IL-12 subunit antibody 4D6 for 20 minutes. Cells were washed again and resuspended in 100 .mu.1 of a 5 .mu.g/**ml** streptavidin-phycoerythritin conjugate (Fisher Biotech) for 15 minutes. The cells were then washed again before analysis on a FACScan flow cytometer. . .

DETDESC:

DETD(89)

RNA . . . F. Fritsch, T. Maniatis, Cold Spring Harbor Laboratory Press 1989 (hereinafter "Molecular Cloning Manual")). The RNA blots were hybridized (7.times.10.sup.5 cpm/**ml**, 30 **ml**) with labeled probe in 5.times.SSC (1.times.SSC =0.15 M NaCl-0.015 M NaCitrate)-50% formamide-5.times.Denhardts solution (1.times.Denhardts=0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, 0.02% bovine serum albumin fraction V) -0.3% SDS-250 .mu.g/**ml** denatured salmon sperm carrier DNA at 37.degree. C. overnight. The probe was generated by random-primer labeling gel-isolated insert from IL-12. . .

DETDESC:

DETD(91)

From . . . in 10 mM Tris-HCl pH 7.8-1 mM EDTA-100 mM NaAcetate. BstXI linked cDNA was applied to the column and 0.5 **ml** fractions were collected. A small aliquot of each fraction was run on a 1% agarose gel,

the gel was dried. . . . above in 60 .mu.l of ligation buffer (50 mM Tris-HCl pH 7.8-10 mM MgCl.sub.2 -10 mM DTT-1 mM rATP -25 .mu.g/**ml** bovine serum albumin) at 15.degree. C. overnight. The following day, the ligation reaction was extracted with phenol, 6 .mu.g of. . . . repeated, followed by a wash with 80% ethanol. Finally, the pellet was dissolved in 6 .mu.l of water and 1 **ml** aliquots were subsequently electroporated into E. coli strain DH-10B (BRL). By electroporating 5 parallel aliquots in this fashion, a library. . .

DETDESC:

DETD(93)

The . . . and were grown up overnight. The next day, the colonies from each pool were scraped off into a separate 50 **ml** aliquot of LB +amp and the cultures were grown for another two hours before plasmid DNA was extracted using QIAGEN. . . pool. The monoclonal anti IL-12 receptor antibody 2-4E6 as discussed above was subsequently bound to the cells in suspension (10 .mu.g/**ml** in PBS- 0.5 mM EDTA-0.02% Na Azide-5% FCS, 1 hour, on ice). The cell suspension was then spun through a. . . added to one bacterial petri dish (9 cm diameter) that had been coated with polyclonal goat anti mouse IgG (20 .mu.g/**ml** in 50 mM Tris-HCl pH 9.5, RT/OVERNIGHT(ON)) and blocked with 1% BSA in PBS (37 degree C./1 hour). COS cells. . . then gently washed off with PBS and the remaining adherent cells in the dishes lysed by the addition of 0.8 **ml** of Hirt lysis solution (0.6% SDS-10 mM EDTA). After transferring to Eppendorf tubes, the lysates were made 1 M NaCl,. . .

DETDESC:

DETD(94)

From . . . slides (2 slides per pool). 2-3 days after transfection, to one of the slides was bound labeled human IL-12 (10.sup.6 cpm/**ml**=300 pM in RPMI 1640 plus 5% FCS for 2-3 hours at 4 degrees C.) and to the other slide labeled monoclonal Ab 2-4E6 (2.times.10.sup.6 cpm/**ml**=1 nM in RPMI 1640 plus 5% FCS for 1 hour at RT). The slides were washed in PBS, fixed for. . . a 100 fold molar excess of cold IL-12 (=nonspecific binding). Wells were washed and the bound radioactivity eluted with 0.5 **ml** of 1% SDS and counted in a gamma counter. Two positive pools were identified in this manner, one representing column. . .

DETDESC:

DETD(98)

Cloned . . . Cells were washed in PBS and lysed in CHAPS lysis buffer (10 mM CHAPS-300 mM NaCl-50 mM Tris-HCl pH 7.4-2 mg/**ml** Iodoacetamide-0.17 mg/**ml** PMSF), precleared by incubation with protein G Sepharose beads (50 .mu.l packed beads per **ml**, Genex) and normal mouse serum (25% final **concentration**) at 4.degree. C. overnight. The beads were spun out and labeled IL-12 receptor was specifically immunoprecipitated from the cleared lysates by adding 5 .mu.g of 2-4E6 antibody per **ml** of sample. The antibody was diluted in PBS containing 1% bovine serum albumin and had been loaded onto 50 .mu.l. . .

DETDESC:

DETD(102)

The . . . anti-COS cell antibodies in the various rat antisera were assessed by flow cytometry as follows. Untransfected COS cells (10.sup.6 cells/0.1 **ml** of Dulbecco's PBS containing 2% heat-inactivated FCS and 01% sodium azide) were preincubated with 400 .mu.g/**ml** normal rat IgG

(Sigma, St. Louis, Mo.) for 15 min. on ice, and then with the indicated amount of rat serum for 30 min. on ice. The cells were washed and further incubated with 2 .mu.g/**ml** FITC-conjugated F(ab').sub.2 mouse anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) for 30 min. on ice. The cells were. . .

DETDESC:

DETD(104)

To . . . the anti-IL-12R) on lymphoblast proliferation induced by IL-12, IL-2, or IL-7. The concentrations of IL-12, IL-2, and IL-7 were 0.25 **ng/ml**, 1.25 **ng/ml**, and 0.4 **ng/ml**, respectively. These concentrations were chosen because they resulted in similar levels of .sup.3 H-TdR incorporation and were on the steep. . .

DETDESC:

DETD(114)

Metabolic . . . at >200 KDa (FIG. 17, lanes 1,3 and 4; arrow indicates uncrosslinked IL-12). Crosslinking at 2 nM .sup.125 I-IL-12 (a **concentration** equivalent to the KD) gave identical results (not shown). The size of a complex composed of one receptor subunit and. . .

TITLE: Recombinant DNA encoding human receptor for interleukin-12
US PAT NO: **5,536,657** DATE ISSUED: Jul. 16, 1996
[IMAGE AVAILABLE]
APPL-NO: 08/248,532 DATE FILED: May 31, 1994
REL-US-DATA: Continuation-in-part of Ser. No. 94,713, Jul. 19, 1993, abandoned.

SUMMARY:

BSUM(4)

Interleukin-12 (IL-12), formerly known as cytotoxic lymphocyte maturation factor (CLMF) or natural killer cell stimulatory factor (NKSF), is a cytokine that has. . .

SUMMARY:

BSUM(5)

IL-12 is a heterodimeric molecule with an approximate molecular weight of about 75 kDa consisting of two disulfide-linked subunits: p35, having an approximate molecular weight of about 35 kDa, and p40, having an approximate molecular weight of about 40 kDa, (2, 4-6). The p40 subunit shares amino acid sequence homology with the interleukin-6 receptor (IL-6R) (7) and therefore belongs to. . . been speculated that the p35/p40 heterodimer could represent a cytokine (p35) and soluble cytokine receptor (p40) complex, with the cellular IL-12 receptor providing function analogous to the IL-6 signal transducing protein, gp130 (7, 8).

SUMMARY:

BSUM(6)

The biological activity of IL-12 is mediated by the binding of the intact IL-12 molecule to plasma membrane receptors on activated T or NK cells (9,10); however, the contributions of the individual subunits to receptor binding and signal transduction remain unknown. Studies with neutralizing antibodies to human IL-12 (11) and site-specific chemical modification (12) suggested that the p40 subunit contains epitopes important for IL-12 binding to its receptor. Also, studies with human/mouse chimeric molecules indicated that p35 is responsible for the species specificity of. . .

SUMMARY:

BSUM(7)

We investigated both the binding and biological activities of each IL-12 subunit. COS cells transfected with only the p40 cDNA produced both p40 monomer and p40 homodimer having an approximate molecular weight of about 80 kDa, the latter capable of binding to the IL-12 receptor but unable to mediate cellular proliferation. The 80 kDa p40 homodimer acts as a receptor antagonist useful in regulating the biological activity of IL-12 in immune responses.

DRAWING DESC:

DRWD(2)

FIG. 1A and 1B. Dose-response binding of human IL-12 and COS-expressed rp40 to KIT225/K6 cells analyzed by flow cytometry. Varying concentrations of purified human IL-12 or rp40 containing conditioned medium (determined by EIA (enzyme immunoassay) using IL-12 as standard) were incubated with KIT225/K6 cells and detected with biotinylated 8E3 mAb followed by streptavidin-PE as described in the. . . incubated only with biotinylated-8E3 and streptavidin-PE. Curves b and c represent cells incubated with 100 and 500 ng/ml of human IL-12, respectively. FIG. 1B: curve a represents nonspecific staining, and curves b, c, d, and e represent cells incubated with 2.5,. . .

DRAWING DESC:

DRWD(3)

FIGS. 2A, 2B, 2C and 2D. Specificity of rp40 binding to KIT225/K6 cells detected by FACS analysis. Purified human IL-12 (FIG. 2A), conditioned media from cultures of COS cells cotransfected with human p35 and p40 cDNAs (FIG. 2B), or with. . . p40 cDNA alone (FIG. 2C) were diluted to 0.5 .mu.g/ml (determined by EIA) and incubated with 4A1 neutralizing monoclonal anti-human IL-12 antibody (b) or with normal rat IgG (R-IgG) (c) at a final concentration of 25 g/ml at room temperature for. . .

DRAWING DESC:

DRWD(5)

FIGS. . . . rp40 and rp35/rp40 heterodimer proteins. Conditioned media (0.5 ml) were immunoprecipitated with 5 .mu.g IgG protein isolated from goat anti-human IL-12 antisera, separated by SDS/PAGE under nonreducing (FIG. 4A) or reducing (FIG. 4B) conditions and analyzed by immunoblot using rabbit anti-human IL-12 antisera and peroxidase-conjugated donkey anti-rabbit IgG. Samples loaded to each lane were as indicated. Human IL-12 from CHO cells was loaded with two different doses (50 ng and 200 ng, respectively) for comparison. Positions of molecular. . .

DRAWING DESC:

DRWD(6)

FIGS. 5A and 5B. Deglycosylation of COS-expressed human rp40 proteins. Purified human IL-12 (0.5 .mu.g) and COS-expressed human rp40 proteins immunoprecipitated with goat anti-human IL-12 antisera were deglycosylated by N-deglycosidase F as described in Materials and Methods. Duplicate samples of the deglycosylated proteins were separated. . .

DRAWING DESC:

DRWD(7)

FIGS. . . . in the p40 EIA and the KIT225/K6 FACS binding assay. The EIA data (-.largecircle.-) were plotted as .mu.g/ml (using human IL-12 as a standard), and the binding data (-.circle-solid.-) were plotted as the mean peak of fluorescence intensity (FIG. 6A). The. . .

DRAWING DESC:

DRWD(8)

FIG. 7. Inhibition of [.sup.125 I]human IL-12 binding to human PHA-blasts by COS-expressed rp40 proteins. Varying concentrations of purified human IL-12 heterodimer (-.circle-solid.-), COS-expressed rp40 homodimer (-.largecircle.-) or rp40 monomer (-.box-solid.-) (determined by EIA using IL-12 as standard) were incubated with 1.times.10.sup.6 PHA-blasts in the presence of 100 pM [.sup.125 I]human IL-12 for 1.5 h at room temperature. The data represent specific binding of [.sup.125 I]IL-12 and are expressed as percentage of the amount of [.sup.125 I]IL-12 bound to the cells in the presence of the indicated concentration of unlabeled IL-12 or rp40 proteins when compared with the total specific binding in the absence of unlabeled IL-12.

DRAWING DESC:

DRWD(9)

FIG. 8. COS-expressed human p40 homodimer induces little proliferation of human PHA-blasts. Serial dilutions of purified native human IL-12 (-.largecircle.-), partially purified COS-expressed human rp40 homodimer (-.circle-solid.-), or PBS buffer (--) were incubated with 2.times.10.sup.4 PHA-blasts. Proliferation was measured. . . assay as described in Materials and Methods. The concentration of rp40 was determined by a sandwich EIA using native human IL-12 as standard as described in Materials and Methods.

DRAWING DESC:

DRWD(10)

FIG. 9. Inhibition of IL-12 bioactivity by COS-expressed p40 homodimer. Varying concentrations of COS-expressed human rp40 homodimer were mixed with 0.1 ng/ml of native human IL-12 prior to incubation with 2.times.10.sup.4 PHA-blasts. Neutralization of IL-12 bioactivity by COS-expressed p40 homodimer was measured in a 48 h proliferation assay as described in Materials and Methods. The. . . of an equivalent dilution of PBS buffer. The concentration of p40 was determined by a sandwich EIA using native human IL-12 as standard as described in Materials and Methods.

DRAWING DESC:

DRWD(11)

FIGS. 10A and 10B. Models of IL-12 p35/p40 heterodimer and p40/p40 homodimer binding to the IL-12 receptor and signal transduction. The IL-12 p40 subunit has to be associated with the p35 subunit or with another p40 molecule for proper conformation of the epitopes required for binding to the IL-12 receptor. However, only the heterodimer (FIG. 10A); not the homodimer (FIG. 10B) acts as a full agonist to induce signaling.

DETDESC:

DETD(3)

The IL-12 p40 homodimer is useful as an IL-12 antagonist to block the biological activity of IL-12 in pathologic immune responses. Current evidence from both in vitro and in vivo studies suggest that IL-12 plays an important role in the development of Th1-type helper T cells which promote cell-mediated immune responses (22,27), in triggering. . . production of gamma interferon (32-34) may be involved in the pathogenesis of some autoimmune disorders and septic shock, indicating that IL-12 p40 homodimer should be useful in the treatment of disorders such as rheumatoid and other inflammatory arthritides, Type I diabetes mellitus, multiple sclerosis, systemic lupus erythematosus, septic shock, etc. In addition, IL-12 p40 homodimer should be useful in preventing or delaying homograft rejection and graft versus host disease. In using IL-12 p40 homodimer to prevent or reverse pathologic immune responses, it can be combined with other cytokine antagonists such as antibodies. . .

DETDESC:

DETD(4)

The . . . general, appropriate dosages are those which are large enough to produce the desired effect, for example, blocking the binding

of IL-12 to its receptor. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions,. . .

DETDESC:

DETD(9)

KIT225/K6, . . . cell line KIT225 (14), was obtained from Dr. T. Waldmann, NIH/NCI (Bethesda, Md.). These cells were previously found to express IL-12 receptors (15). KIT225/K6 cells were cultured in RPMI 1640 medium (BioWhittaker, Walkersville, Md.) supplemented with 2 mM L-glutamine (Sigma, St.. . .

DETDESC:

DETD(10)
Expression of IL-12 subunits.

DETDESC:

DETD(11)

The IL-12 expression constructs for COS-expression were built in the pEF-BOS vector which contains the promoter of the human polypeptide chain elongation. . .

DETDESC:

DETD(12)

The human IL-12 p40 construct for expression in Baculovirus system was built in pACDZ-1 vector (16b) (obtained from R. Gentz, F. Hoffmann-La Roche. . . p40 expressing plasmid pACDZ-1. Limited dilution cloning in microtiterplates was used to isolate a single recombinant baculovirus expressing the human IL-12 p40 subunit.

DETDESC:

DETD(13)
IL-12 receptor binding and proliferation assays.

DETDESC:

DETD(14)

The binding of COS-expressed IL-12 molecules to IL-12 receptor-bearing cells was measured by FACS (fluorecense activated cell sorthing) analysis essentially as described by Desai et al.(10). Briefly, 1.times.10.sup.6 KIT225/K6 cells suspended in 25 .mu.l of FACS buffer (PBS (phosphate-buffered-saline)/2% FCS/0.05% sodium azide) were incubated with IL-12 preparations (25.mu.l) at room temperature for 40 min, followed by incubation with biotinylated mAb 8E3 (5 .mu.g/ml, 50 .mu.l), (11). . . The stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson). Specificity of binding was determined by preincubating the IL-12 preparations (0.5 .mu.g/ml) with 4A1 (25 .mu.g/ml), a rat inhibitory anti-human IL-12 monoclonal antibody, prior to adding cells. Control samples were incubated with normal rat IgG (25 .mu.g/ml). The receptor binding properties of the COS-expressed IL-12 molecules were also evaluated in an [.sup.125 I]IL-12 competitive receptor binding assay performed essentially as previously described (11). 0.1 ml aliquots of serial dilutions of culture supernatant fluids or purified IL-12 were mixed with 0.05 ml aliquots of binding buffer (RPMI-1640, 5% FCS, 25 mM HEPES pH 7.4) containing [.sup.125 I]IL-12 (2.times.10.sup.6 cpm). The mixture was added to 0.1 ml of activated blasts (1.times.10.sup.7 cell/ml) and

incubated in a shaking water bath at 25.degree. C. for 1.5 h. Non-specific binding was determined by inclusion of 20 .mu.g/ml unlabeled IL-12 in the assay. Incubations were carried out in duplicate. Cell bound radioactivity was separated from free [125I]IL-12 by centrifugation of 0.1 ml aliquots of the assay contents in duplicate through 0.1 ml silicone oil for 90 sec. . . .

DETDESC:

DETD(15)

The biological activity of COS-expressed IL-12 molecules was evaluated in proliferation assays using 4-day PHA-activated human lymphoblasts previously described (4, 13).

DETDESC:

DETD(16)
Anti-IL-12 antibodies and sandwich enzymatic immunoassay (EIA).

DETDESC:

DETD(17)

Goat and rabbit anti-human IL-12 antisera were obtained from animals immunized with purified human rIL-12 that had been expressed in CHO cells (kindly provided by. . . 100 ml of the antisera by Protein-G Sepharose (Pharmacia LKB, Piscataway, N.J.) affinity chromtography according to the manufacturer's procedures. Anti-human IL-12 antibodies were purified from the IgG fractions on a human IL-12-conjugated hydrazide AvidGel F. (BioProbe International) immunoaffinity column (1.5.times.2.0 cm, 0.55 mg protein per ml resin). Biotinylation of the antibodies using Biotin X-NHS (Calbiochem, San Diego, Calif.) was performed as described previously (18). Monoclonal antibodies 4A1 and 8E3 are rat antibodies specific for the p40 subunit of human IL-12 (11) (kindly provided by Dr. Richard Chizzonite, Hoffmann-La Roche Inc., Nutley, N.J.).

DETDESC:

DETD(18)

The IL-12 sandwich EIA, using mAb 4A1 as a capture antibody and peroxidase-conjugated 8E3 as detection antibody, was performed as described previously (11). This assay detects IL-12 heterodimer and p40 subunit but not p35 subunit. Therefore, a second IL-12 sandwich EIA using polyclonal antibodies was developed to detect both p40 and p35. In this assay, 96 well EIA plates (Nunc MaxiSorp, Thousand Oaks, Calif.) were coated with affinity-purified goat anti-human IL-12 antibody (2 .mu.g/ml, 50 .mu.l/well) at 4.degree. C. overnight and blocked with 1% BSA in PBS pH 7.4 for 1 h at RT. Serial dilutions of IL-12 and culture supernatant fluids were applied to the plates, and incubated at RT for 2.5 h. The plates were subsequently incubated with biotinylated, affinity-purified rabbit anti-human IL-12 antibody (500 ng/ml, 50 .mu.l/well), followed by peroxidase-conjugated streptavidin (1 .mu.g/ml, 50 .mu.l/well, Sigma, St. Louis, Mo.). Color was developed. . . nm was determined with a Vmax Kinetic. Microplate reader (Molecular Devices, Palo Alto, Calif.). All values are based on an IL-12 standard curve with no corrections calculated for differences in molecular weights of monomers or dimers.

DETDESC:

DETD(20)

Immunoprecipitation of COS-expressed IL-12 subunits and heterodimers was performed as described (17). Briefly, 0.5 ml supernatant fluids from transfected COS cultures were incubated with 5 .mu.g IgG protein isolated from goat anti-IL-12 antiserum at 4.degree. C. on a rotating mixer overnight. The immune complexes were adsorbed onto Protein G-Sepharose (50% suspension, 10. . .

DETDESC:

DETD(22)

SDS-PAGE . . . by incubation in PBST buffer (PBS with 0.05% v/v Tween-20) containing 5% (w/v) non-fat dry milk, and then probed with anti-IL-12 rabbit antisera (1:500 dilution). After three washes with PBST buffer, the membranes were incubated at room temperature with peroxidase-conjugated donkey. . .

DETDESC:

DETD(26)

500 ng of pure human IL-12 or immunoprecipitated rp40 protein was denatured by heating at 95.degree. C. for 5 rain in 0.25M Na.sub.2 HPO.sub.4 (pH 7.2),. . .

DETDESC:

DETD(28)

The . . . 10% non-reducing SDS gel and transferred electrophoretically to an Immobilon.TM. PVDF membrane (Millipore, Bedford, Mass.). The bands at .about.80 and .about.40 kDa identified by Coomassie blue staining were subjected to automated Edman degradation on an Applied Biosystems Model 470A gas-phase sequencer with. . .

DETDESC:

DETD(30)
Expression and characterization of human IL-12 subunits.

DETDESC:

DETD(31)

Human IL-12 subunits p35 and p40, or human IL-12 p35/p40 heterodimer were expressed by transfecting either subunit cDNA independently or cotransfecting both cDNAs at a 1:1 (w:w) ratio in COS cells. Secretion of the recombinant proteins was evaluated by two different EIA's. The p40-specific monoclonal antibody-based EIA was capable of detecting the p40 subunit and the p40/p35 heterodimer. The IL-12 specific polyclonal EIA was also capable of detecting the p35 subunit. Using human IL-12 as a standard, the concentration range of rp40 and rp35/rp40 proteins in the conditioned media was 0.5-3.0 .mu.g/ml, whereas the. . .

DETDESC:

DETD(32)

The COS-expressed human IL-12 recombinant proteins were initially examined for their ability to inhibit the binding of [.sup.125 I]human IL-12 to PHA-activated human lymphoblasts. The rp40 supernatants at a 1:2 dilution exhibited 30-40% inhibition of [.sup.125 I]IL-12 binding in three independent experiments, whereas the

rp35 supernatants were inactive. The binding of rp40 to the IL-12 receptor was further characterized by flow cytometry using KIT225/K6 cells which constitutively express IL-12 receptors (IL-12R) (15). Dose-dependent binding of human IL-12 and rp40 to KIT225/K6 was observed in the range of 2.5-500 ng/ml (FIGS. 1A and 1B). Specificity of the binding was demonstrated by achieving greater than 80% inhibition of the binding by preincubation of IL-12 or rp40 with an inhibitory rat anti-human p40 monoclonal antibody, 4A1 (FIGS. 2A, 2B, 2C and 2D). Normal rat IgG had no effect on IL-12 or rp40 binding.

DETDESC:

DETD(33)

Conditioned media containing the COS-expressed IL-12 subunit proteins were evaluated in the human PHA-blast proliferation assay (FIG. 3). The rp35/rp40-containing medium supported T cell proliferation in. . .

DETDESC:

DETD(34)
Characterization of the rp40 40 kDa and 80 kDa species.

DETDESC:

DETD(35)

The recombinant human IL-12 subunits were immunoprecipitated with anti-human IL-12 goat antiserum and characterized by SDS-PAGE and Western blot analysis. Analysis of the rp40 expressed by COS cells transfected with. . . and converted the reduced triple bands to a single 36 kDa product similar to p40 subunit of the deglycosylated human IL-12 (12) demonstrating that the multiple bands of rp40 expressed in COS cells are due to glycosylation heterogeneity.

DETDESC:

DETD(36)

In contrast, the immunoprecipitation of rp35 protein revealed only a single band with a molecular weight of 35 kDa under reducing conditions (FIG. 4B). Under nonreducing conditions, a set of lightly stained bands were found at 60-70 kDa suggesting that rp35 may also partially form dimers. However, the polyclonal goat anti-IL-12 antibody poorly recognized the rp35proteins (FIG. 4A). Coexpression of p35 and p40 gave a pattern of bands which was a. . .

DETDESC:

DETD(37)

To . . . band at .about.80 kDa and two bands at 35-40 kDa gave NH.sub.2 -terminal sequences identical to that of native human IL-12 p40 purified from NC-37 cells (4, 12) (Table I). No trace of p35 sequences as identified with the rp40 species.. . .

DETDESC:

DETD(38)

TABLE I

Amino-terminal Sequences of COS-expresssed Human p40 Monomer, p80 Homodimer and Native Human IL-12 p40 subunit

Protein	Sequence
Native Human p40	I W E L K K D V Y V.sup.a [SEQ. . .

DETDESC:

DETD(39)

The . . . by Superalex-75 gel filtration chromatography. Two EIA positive protein peaks were identified at molecular weights corresponding to 80 kDa and 40 kDa (FIG. 6A). SDS-PAGE and Western blot analysis of the fractions confirmed the separation of dimer from monomer rp40 (FIG. 6B).. . .

DETDESC:

DETD(40)

The . . . FACS analysis. Binding activity correlated only with the 80 kDa p40-EIA positive protein (FIGS. 6A and 6B). The 80 and 40 kDa peak fractions were pooled separately, concentrated and examined in the competitive radioligand receptor binding assay (FIG. 7). The 80 kDa protein pool inhibited [.sup.125 I]human IL-12 binding to PHA-blasts with an IC.sub.50 of 80 ng/ml, which is similar to the IC.sub.50 of human IL-12 heterodimer (20 ng/ml). However, the slope of the competition curve by the 80 kDa homodimer differed from that of IL-12 heterodimer suggesting a different binding interaction with the receptor. The 40 kDa protein pool inhibited [.sup.125 I]human IL-12 binding with an IC.sub.50 about one hundred times higher, which was probably due to a small amount of contamination with. . .

DETDESC:

DETD(41)

The . . . 8). No proliferative response was observed with either rp40 species even at concentrations 10,000 times higher than that of human IL-12 required to elicit a 50% maximum response. The rp40 dimer was tested for its ability to neutralize IL-12-dependent proliferation of PHA-blasts. The 80 kDa protein at varying concentrations was mixed with 0.1 ng/ml of human IL-12 and added to PHA-blasts. Dose-dependent inhibition of IL-12-induced proliferation of PHA-blasts was achieved with an IC.sub.50 of .mu.g/ml (FIG. 9).

DETDESC:

DETD(42)

IL-12 is unique among the lymphokines and cytokines in that it is a heterodimeric protein. Previous studies suggested that the p40. . .

DETDESC:

DETD(43)

To . . . of the rp40 by immunoaffinity chromatography and HPLC gel permeation chromatography revealed that the 80 kDa protein, but not the 40 kDa protein bound to the IL-12R.

DETDESC:

DETD(44)

Unfortunately, no reagents were available to distinguish an 80 kDa p40 homodimer from the 75 kDa IL-12 heterodimer. The possibility that the 80 kDa protein was not a homodimer of p40 but a heterodimer consisting of one IL-12 p40 subunit and a second 35-40 kDa exogenous COS-derived protein was investigated. In particular, reports that many cell lines constitutively express IL-12 p35 mRNA (21) raised the possibility that the 80 kDa protein could be human IL-12 p40 associated with COS-derived IL-12 p35. Western blot analysis by using p35 specific antibody and deglycosylation experiments (FIGS. 5A and 5B) supported the notion that. . . monomer. The lack of bioactivity despite good binding activity further suggested that the second protein was not a COS-derived p35 IL-12 subunit (assuming no species restriction in the activity of monkey IL-12 on human cells). Also, expression of p40 in a baculovirus system yielded a biologically inactive 80 kDa form of p40 capable of binding to the receptor. It seems unlikely that insect cells produce an IL-12-like p35 protein. Most importantly, confirmation of the identity of the 80 kDa protein as p40 homodimer was provided by amino. . .

DETDESC:

DETD(45)

In competitive binding analysis, the p40 homodimer was found to bind to the IL-12R nearly as strongly as heterodimeric IL-12, suggesting that the key binding epitopes of IL-12 are localized in the p40 subunit. Though the IC.sub.50 values for the heterodimer and the homodimer were similar, 20 and. . .

DETDESC:

DETD(46)

The IL-12 p40 subunit has been previously reported to be produced in excess of heterodimeric IL-12 both by activated B lymphoblastoid lines and by human PBMC stimulated to produce IL-12 (12, 23). It is possible that the p40 homodimer is formed in cells expressing p40/p35 heterodimers.

DETDESC:

DETD(47)

Based on our observations on the roles of the IL-12 subunits in binding and signaling, a model of IL-12 binding to its receptor is illustrated in FIGS. 10A and 10B. The p40 subunit contains the receptor binding epitopes that,. . . an agonist to mediate cellular transduction signals (FIG. 10A). In contrast, the p40/p40 dimer behaves as an antagonist to suppress IL-12 mediated responses (FIG. 10B). Gearing and Cosman (7) have suggested that IL-12 is analogous to a complex of cytokine and soluble receptor based on the homology between p40 and the interleukin 6. . . is a gp 130-like signal-transducing protein. Our model of the interaction between p35, p40 and the IL-12R proposes that the IL-12/IL-12R system may function similarly to the IL-6R system. In the latter system, gp130 does not bind IL-6 (24), and neither. . . IL-6 with soluble IL-6R can bind to gp130 to initiate signal transduction (26). Similarly, it appears that the association of IL-12 p40 with p35 results in an alteration in p40 that permits binding to IL-12R with subsequent initiation of p35-dependent, IL-12R-mediated. . .

DETDESC:

DETD(49)
1. . . . F. Sinigaglia, R. Chizzonite, U. Gubler, and A. S. Stern. 1991. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). J. Immunol. 147:874.

DETDESC:

DETD(57)
9. Chizzonite, R., T. Truitt, B. B. Desai, P. Nunes, F. J. Podlaski, A. S. Stern, and M. K. Gately. 1992. IL-12 receptor. I. Characterization of the receptor on phytohemagglutinin-activated human lymphoblasts. J. Immunol. 148:3117.

DETDESC:

DETD(58)
10. Desai, B. B., P. M. Quinn, A. G. Wolitzky, P. K. A. Mongini, R. Chizzonite, and M. K. Gately. 1992. IL-12 receptor. II. Distribution and regulation of receptor expression. J. Immunol. 148:3125.

DETDESC:

DETD(59)
11. . . . Truitt, F. J. Podlaski, A. G. Wolitzky, P. M. Quinn, P. Nunes, A. S. Stern, and M. K. Gately. 1990. IL-12: monoclonal antibodies specific for the 40-kDa subunit block receptor binding and biologic activity on activated human lymphoblasts. J. Immunol. 147:1548.

DETDESC:

DETD(61)
13. . . . C. M. Dwyer, W. McComas, P. C. Familietti, M. K. Gately, and U. Gubler. 1992. Cloning and expression of murine IL-12. J. Immunol. 148:3433.

DETDESC:

DETD(69)
21. . . . D. Seiburth, B. Perussia, J. Yetz-Adalpe, A. D'Andrea, and G. Teinchieri. 1992. Cell sources of natural killer cell stimulatory factor (NKSF/IL-12) transcreipts and subunit expession. FASEB J. 6:A1335.

DETDESC:

DETD(70)
22. . . . S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of T.sub.H 1 CD4.sup.+ T cells through IL-12 produced by Listeria-induced macrophages. Science 260: 547-549.

DETDESC:

DETD(75)
27. . . . M. G. Giudizi, M.-P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. J. Exp. Med. 177:1199.

DETDESC:

DETD(80)

preparation of CLMF have been identified and characterized by 1: immunoprecipitation of .sup.125 I-labelled CLMF,. . . LAK cell induction assays. Western blot analysis demonstrate that each antibody binds to the 70 kDa heterodimer and to the **40 kDa** subunit. These data demonstrated that the 20 antibodies were specific for CLMF and in particular for the **40 kDa** subunit of CLMF. A CLMF receptor binding assay has been developed to evaluate the ability of individual antibodies to inhibit. . . bioactivity as assessed by the T-cell proliferation and LAK cell induction assays. The ability of the antibodies specific for the **40 kDa** subunit of CLMF to neutralize CLMF bioactivity indicates that determinants on the **40 kDa** subunit are necessary for binding to the CLMF cellular receptor.

SUMMARY:

BSUM(24)

The . . . of natural and recombinant human CLMF, the development of human CLMF immunoassays, the identification of the active site of the **40 kDa** subunit of CLMF and as possible therapeutic treatments which require selective immunosuppression of cytotoxic T cells, such as in transplantation. **Monoclonal antibodies** which recognize different epitopes on human CLMF can be used as reagents in a sensitive two-site immunoassay to measure levels. . .

SUMMARY:

BSUM(25)

The present invention is directed to **monoclonal antibodies** against CLMF which exhibit a number of utilities including but not limited to:

SUMMARY:

BSUM(26)

1. Utilizing the **monoclonal antibodies** as affinity reagents for the purification of natural and recombinant human CLMF;

SUMMARY:

BSUM(27)

2. Utilizing the **monoclonal antibodies** as reagents to configure enzyme-immunoassays and radioimmunoassays to measure natural and recombinant CLMF in biological fluids, cell culture supernatants, cell. . .

SUMMARY:

BSUM(28)

3. Utilizing the **monoclonal antibodies** as reagents to construct sensitive two-site immunoassays to measure CLMF in biological fluids, cell culture supernatants and human cell extracts;

SUMMARY:

BSUM(29)

4. Utilizing the **monoclonal antibodies** as reagents to identify determinants of the **40 kDa** subunit which participate in binding to the **35 kDa** subunit and which participate in binding to the CLMF cellular receptor;

SUMMARY:

BSUM(30)

5. Utilizing the intact IgG molecules, the Fab fragments or the humanized IgG molecules of the inhibitory **monoclonal antibodies** as therapeutic drugs for the selective blockade of proliferation and activation of cytotoxic T cells, such as in transplantation.

DRAWING DESC:

DRWD(8)

FIG. . . . .beta.-mercaptoethanol) and reducing (in the presence of .beta.-mercaptoethanol) conditions showing the 75,000 molecular weight CLMF separated into two subunits of **40 kDa** and **35 kDa**.

DRAWING DESC:

DRWD(14)

FIG. 13 is a schematic diagram showing the separation of the **40 kDa** subunit from the **35 kDa** subunit of the CLMF cytokine of the present invention.

DRAWING DESC:

DRWD(15)

FIG. . . . a schematic diagram showing the determination of the amino acid composition, N-terminal sequencing, proteolytic digestion and complete sequencing of the **40 kDa** subunit of the CLMF cytokine of the present invention.

DRAWING DESC:

DRWD(16)

FIG. 15 is a tryptic peptide map of the digested **40 kDa** subunit of the CLMF cytokine of the present invention.

DRAWING DESC:

DRWD(17)

FIG. 16 is a proteolytic peptide map of the digested **40 kDa** subunit CLMF in which the proteolytic enzyme which was used was Staphylococcus aureus V8 protease.

DRAWING DESC:

DRWD(18)

FIG. 17 is a chart which summarizes the protein structural determination of the **40 kDa** subunit of CLMF.

DRAWING DESC:

DRWD(25)

FIG. 24 is an SDS PAGE of pure CLMF and "free" unassociated **40 kDa** subunit of CLMF purified by Affinity Chromatography according to Example 7.

DRAWING DESC:

DRWD(26)

FIG. 25 shows the DNA and deduced amino acid sequences for the **40 kDa** subunit of CLMF.

DRAWING DESC:

DRWD(27)

FIG. 26 shows the cDNA sequence and deduced amino acid sequence for the human **35 kDa** CLMF subunit.

DRAWING DESC:

DRWD(29)

FIG. 28 shows SDS PAGE analysis of immunoprecipitation of .sup.125 I-CLMF by **monoclonal antibodies** 4A1 (1), 4D1 (2), 8E3 (3), 9C8 (4) and control (5) and by immune rat serum (6 and 8) and. . .

DRAWING DESC:

DRWD(33)

FIGS. 32A and B shows Western blot analysis of the reactivity of monoclonal and rat polyclonal anti-CLMF antibodies with CLMF **40 kDa** subunit under non-reducing conditions (A) and under reducing conditions (B).

DRAWING DESC:

DRWD(36)

FIG. 35 shows the inhibition of .sup.125 I-CLMF binding to PHA-activated PBL blast cells by **monoclonal antibody** supernatants. The data are expressed as % inhibition of .sup.125 I-CLMF binding to the cells in the presence of a. . .

DRAWING DESC:

DRWD(37)

FIG. 36 shows the inhibition of .sup.125 I-CLMF binding to PHA-activated PBL blast cells by various concentrations of purified **monoclonal antibodies**. The data are expressed as the amount (% cpm bound) of .sup.125 I-CLMF binding to the cells in the presence. . .

DRAWING DESC:

DRWD(38)

FIG. . . . shows Western blot analysis of the reactivity of a rabbit polyclonal anti-CLMF antibody with 75 kDa CLMF (nonreduced) and with **35 kDa** CLMF subunit (reduced). The antibody was prepared against a synthetic peptide fragment of the **35 kDa** CLMF subunit.

DRAWING DESC:

DRWD(39)

FIG. 38 shows the effect of **IL-12** on IgE and IFN-.gamma. production by IL-4-stimulated PBMC.

DRAWING DESC:

DRWD(40)
A. PBMC were cultured for 12 days in the presence of 10 ng/ml of IL-4 and increasing concentrations of **IL-12** (0.1 to 100 pM). Shown are the mean.+-.1 SEM of three experiments; *significantly different from control without IL-2 at P<0.01,. . .

DRAWING DESC:

DRWD(41)
B. Cells were cultured in the presence of IL-4 and **IL-12** (60 pM), with or without anti-**IL-12** mAbs (a mixture of antibodies 4A1 and 20C2, each at 10 .mu.g/ml).

DRAWING DESC:

DRWD(42)

FIG. 39 shows that **IL-12** suppresses the accumulation of productive but not germ-like C.epsilon. transcripts. Total RNA was extracted from PBMC cultured for 10 days. . . with 10 ng/ml of IL-4, in the absence (lanes 1, 3 and 5) or in the presence of 60 pM **IL-12** (lanes 2, 4, and 6). Northern blot was performed as described. The membrane was hybridized with .sup.32 P-labeled probes specific. . .

DETDESC:

DETD(3)

The CLMF (or **IL-12**) active proteins of the present invention include the homogeneous natural CLMF protein as well as CLMF proteins which contain a. . .

DETDESC:

DETD(53)
Methods for Assay of **Monoclonal Antibodies**

DETDESC:

DETD(55)

Immunodepletion of CLMF. Hybridoma culture supernatants or purified **monoclonal antibodies** were tested for their ability to immunodeplete CLMF as follows: Goat anti-rat IgG-agarose beads (Sigma Chemical Co., St. Louis, Mo.). . . Aliquots (0.2 ml) of the bead suspension were added to 1.5 ml Eppendorf tubes, together with the indicated amounts of **monoclonal antibodies** or hybridoma supernatant solutions. The volume of each mixture was brought to 1.4 ml by the addition of hybridoma maintenance. . .

DETDESC:

DETD(64)

Therefore, . . . 40,000 and 35,000 daltons (FIG. 7). Thus we may conclude that CLMF is a 75 kDa heterodimer composed of disulfide-bonded **40 kDa** and **35 kDa** subunits.

DETDESC:

DETD(96)

Three . . . nos. 47, 54 and 57) containing four peptides were sequenced. All four peptides were from the amino-terminal region of the **40 kDa** subunit indicating that the N-terminus of the protein is most susceptible to V8-digestion.

DETDESC:

DETD(107)

The . . . acid composition and partial sequence analysis of the lower molecular weight subunit of the CLMF protein. Approximately 1 .mu.g of **35 kDa** subunit was subjected to hydrolysis, and its amino acid composition was determined (Table 7). Proline, cysteine and tryptophan were not. . .

DETDESC:

DETD(124)

Fraction No.	N-Terminal Sequence
47	V--D--A--V--H--K--L--K--Y--E--?--Y--T--S--(S?)--F--F--I--R--D--I--I--K--P-- (Starts at residue #190 of **40 kDa** subunit)

DETDESC:

DETD(128)

An affinity chromatography resin was prepared by covalently attaching 7B2 **monoclonal antibody** to activated agarose. Similarly, the below outlined purification could also be carried out by covalently coupling the antibody to silica. . .

DETDESC:

DETD(137)

Protein . . . were silver stained [Morrissey, Anal. Biochem. 117:307-310] to visualize protein. The acid eluant contained pure CLMF and the "free" unassociated **40 kDa** subunit of CLMF (FIG. 24).

DETDESC:

DETD(153)
Cloning of a cDNA Coding for the **40 kDa** Subunit of Human CLMF

DETDESC:

DETD(158)
3) Use of PCR to Generate a DNA Probe Specific for the **40 KDa** CLMF Subunit cDNA

DETDESC:

DETD(159)

The partial N-terminal sequence of the purified **40 kDa** protein is IWELKKDVYVVELDWYPDAP . . . Two primers for use in mixed primer PCR were designed and synthesized by standard. . . in the same manner, to represent the antisense strand corresponding to the amino acid sequence YPDAP in the partial N-terminal **40 kDa** sequence. The reverse

primer thus has the sequence 5' ctc gaa ttc ngg ngc a/gtc ngg a/gta and is a. . . . was amplified, since the deduced amino acid sequence matches exactly the partial amino terminal amino acid sequence from the purified **40 kDa** protein. This information was subsequently used to design a 54 bp long oligonucleotide probe that could be used for screening. . .

DETDESC:

DETD(164)

A . . . frames coding for tryptic peptides that had actually been isolated from purified 40 Kd protein. The complete sequence of the **40 kDa** subunit as deduced from the cDNA is shown in FIG. 25. The cDNA cods for one open reading frame of. . . Met, followed by another 21 amino acids that make up a classical hydrophobic signal peptide. The N-terminus of mature purified **40 kDa** subunit, i.e. IWELKKD . . . , follows immediately after the signal sequence. The mature protein thus consists of 306. . .

DETDESC:

DETD(166)

Cloning of a cDNA Coding for the **35 kDa** Subunit of Human CLMF

DETDESC:

DETD(167)

Cell culture, isolation of mRNA and establishment of a cDNA library were as described earlier for the cloning of the **40 kDa** subunit.

DETDESC:

DETD(168)
Use of Mixed Primer PCR to Generate a DNA Probe Specific for the **35 kDa** subunit cDNA

DETDESC:

DETD(169)

The partial N-terminal sequence of the purified **35 kDa** subunit is ?NLPVATPDPGMFP?LHHSQNLLRAV . . . Two primers for use in mixed primer PCR were generated by standard procedures. The. . .

DETDESC:

DETD(174)

A . . . a naturally occurring internal EcoRl site. The complete sequence of the cDNA and the deduced amino acid sequence for the **35 kDa** CLMF subunit are shown in FIG. 26. The cDNA codes for an open reading frame of 219 amino acids, starting. . . following 21 amino acids constitute a classical hydrophobic signal sequence. Immediately following the signal peptide, the N-terminus of the mature **35 kDa** protein starts with the sequence RNLPVAT . . . Purified **35 kDa** protein had yielded the sequence ?NLPVAT . . . The mature **35 kDa** protein thus consists of 197 amino acids, containing three possible N-linked glycosylation sites and 7 cys-residues. The molecular weight of. . .

DETDESC:

DETD(178)
40 kDa Subunit

DETDESC:

DETD(179)

The two EcoRl fragments constituting the full length cDNA for the **40 kDa** CLMF subunit were ligated to an expression vector similar to pBC12 [See B. Cullen, Meth. Enzymology 152, 684,703, (1987)], except. . . to each other were selected by colony hybridization with a synthetic oligonucleotide that spans the internal EcoRl site in the **40 kDa** cDNA. This oligonucleotide has the following sequence: 5'CTG AAG CCA TTA AAG AAT TCT CGG CAG GTG 3'. It was. . . a primer specific for sequences in the SV 40 early promoter and as reverse primer an oligonucleotide corresponding to the **40 kDa** cDNA sequence positions no. 851-868. Clones with the correct orientation will give a PCR amplicon of 885 bp. Eight out. . .

DETDESC:

DETD(180)
35 kDa Subunit

DETDESC:

DETD(181)

The full length cDNA for the **35 kDa** subunit was amplified out of the original lambda phage by PCR, using primers situated to the left and right of. . . of the cDNA. One clone was chosen for further study after ascertaining its structure by PCR as above for the **40 kDa** construct.

DETDESC:

DETD(183)

The DNAs for the expression constructs of the **40 kDa** and **35 kDa** subunits were introduced into COS cells on 6 cm diameter plates by the DEAE Dextran transfection procedure (7.times.10.sup.5 cells/dish plated;. . .

DETDESC:

DETD(184)

Supernatant fluids from cultures of COS cells which had been transfected with cDNA encoding the **35 kDa** CLMF subunit or the **40 kDa** CLMF subunit or with both cDNAs were tested for CLMF activity in the T cell growth factor assay (Table 12).. . .

DETDESC:

DETD(185)
. . .
39,180 .+-. 545
Natural CLMF 1.6 units/ml
25,996 .+-. 763
Supernatant fluid from
cultures of COS cells
transfected with:
35 kDa CLMF subunit cDNA
1/5 dilution
15,332 .+-. 797

35 kDa CLMF subunit cDNA
1/25 dilution
12,149 .+-. 379
40 kDa CLMF subunit cDNA
1/5 dilution
14,883 .+-. 1039
40 kDa CLMF subunit cDNA
1/25 dilution
13,889 .+-. 110
35 kDa + **40 kDA** CLMF subunit cDNAs
1/5 dilution
66,228 .+-. 166
35 kDa + **40 kDA** CLMF subunit cDNAs
1/25 dilution
47,873 .+-. 275
Mock transfected 1/5 dilution
14,368 .+-. 628
Mock transfected. . .

DETDESC:

DETD(189)

Isolation and Identification of **Monoclonal Antibodies** Specific for CLMF. Serum isolated at the 3rd bleed from the rat immunized with partially purified CLMF (Table 13) neutralized. . .

DETDESC:

DETD(190)

The radioiodinated partially purified CLMF preparation contains predominantly the CLMF 75 kDa heterodimer, a small amount of the free CLMF **40 kDa** subunit and two other proteins of approximately 92 kDa and 25 kDa (FIG. 28). The .sup.125 I-labelled CLMF preparation retained. . . alter the configuration of the CLMF molecule. The CLMF immunized rat serum immunoprecipitated the 75 kDa heterodimer and the free **40 kDa** subunit (Lanes 6 and 8, FIG. 28) wheras normal rat serum did not immunoprecipitate these radiolabelled proteins (Lanes 7 and 9, FIG. 28). Four individual **monoclonal antibodies** also immunoprecipitated the 75 kDa heterodimer and the free **40 kDa** subunit (FIG. 28) but did not immunoprecipitate the 92 kDa or 25 kDa labelled proteins. The immunoprecipitation assay identified twenty hybridomas which secreted anti-CLMF antibodies (Table 14). All the antibodies immunoprecipitated the radiolabelled 75 kDa heterodimer and the free **40 kDa** subunit as determined by SDS/PAGE and autoradiography (data shown for 4 representative antibodies in FIG. 28).

DETDESC:

DETD(193)

Identification of the CLMF Subunit Bound by the **Monoclonal Antibodies**. CLMF is a 75 kDa heterodimer protein composed of **40 kDa** and **35 kDa** subunits. Western blot analysis was used to determine if the monoclonal anti-CLMF antibodies recognized the **40 kDa** or the **35 kDa** subunits. Highly purified 75 kDa CLMF heterodimer was separated by non-reducing SDS/PAGE and transferred to nitrocellulose membrane (FIG. 31). In addition, purified CLMF, which was composed of approximately 95% free **40 kDa** subunit and 5% 75 kDa heterodimer, was separated by both non-reducing and reducing SDS/PAGE and the proteins were transferred to nitrocellulose membrane (FIG. 32). Individual nitrocellulose strips containing the non-reduced 75 kDa CLMF heterodimer (FIG. 31), the non-reduced **40 kDa** subunit (top panel FIG. 32) and the reduced **40 kDa** subunit (bottom panel FIG. 32)

were probed with monoclonal anti-CLMF antibodies, control **monoclonal antibody**, rat anti-CLMF serum and control rat serum. The monoclonal anti-CLMF and rat polyclonal anti-CLMF antibodies bind specifically to an approximately. . . preparations do not show this binding activity (FIG. 31). All the monoclonal and rat polyclonal anti-CLMF antibodies recognize the non-reduced **40 kDa** subunit (FIG. 32A). However, only the rat polyclonal antiserum and three **monoclonal antibodies**, 8E3, 9F5 and 22E7, bind to reduced **40 kDa** subunit protein (FIG. 32B). These data demonstrated that all the **monoclonal antibodies** were specific for the **40 kDa** subunit of CLMF.

DETDESC:

DETD(194)

Identification . . . The previous data demonstrated that the monoclonal anti-CLMF antibodies immunoprecipitated .sup.125 I-labelled CLMF, immunodepleted CLMF bioactivity and bound to the **40 kDa** subunit of CLMF. However, the antibodies present in the hybridoma supernatant solutions could not be directly tested for their ability. . . LAK cell induction assays due to non-specific inhibitory effects of supernatant solutions containing control antibodies. Our previous work with IL-2 **monoclonal antibodies** demonstrated that antibodies which would block .sup.125 I-IL-2 binding to IL-2 receptor bearing cells would also neutralize IL-2 bioactivity. Since. . .

DETDESC:

DETD(199)

TABLE 14

Monoclonal Anti-CLMF Antibodies (**40 kDa** Subunit Specific)
Western Blot.sup.1
.sup.125 I-CLMF/Receptor Assay
Neutralization
Antibody
Red N.R. (% Inhibition).sup.2
of Bioactivity.sup.3
. . . and Red. is reduced
SDS/PAGE For
the western blots, a CLMF sample containing 5% 75 kDa heterodimer and 95%
free **40 kDa** subunit were separated on 10% SDS/PAGE and western blots
prepared as described in methods. The blots were scored as. . .

DETDESC:

DETD(203)

A peptide, comprising amino acids 3-13 of the NH.sub.2 -terminal sequence of the **35 kDa** CLMF subunit and a COOH-terminal cysteine (L-P-V-A-T-P-D-P-G-M-F-C), was synthesized by solid-phase peptide methodology, purified by HPLC, and conjugated to keyhole. . .

DETDESC:

DETD(205)

A . . . of the rabbits (first bleed) was tested by Western blot analysis for reactivity with 75 kDa CLMF and with the **35 kDa** CLMF subunit (FIG. 37). Partially purified CLMF (approximately 120 .mu.g/ml) was run on SDS-PAGE, transferred to nitrocellulose, and treated with. . . streptavidin. The anti-CLMF peptide antibody was found to react both

with nonreduced 75 kDa CLMF protein and with the reduced **35 kDa** CLMF subunit (FIG. 37).

DETDESC:

DETD(206)

Although the antibodies produced in this example were polyclonal, a similar approach could be used to prepare **monoclonal antibodies** to the **35 kDa** subunit of CLMF. The synthetic peptide used in this example or other synthetic peptides based on the amino acid sequence of the **35 kDa** CLMF subunit (FIG. 26) could be used to immunize rats. Fusions could be performed and hybridoma cultures screened for the. . .

DETDESC:

DETD(209)

Synthesis . . . agents such as viruses. Also known as NKSF (Natural Killer Cell Stimulatory Factor) or as CLMF (Cytotoxic Lymphocyte Maturation Factor) **IL-12** is a 75 kDa heterodimeric glycoprotein displaying several in vitro activities including: (a) the enhancement, in synergy with IL-2, of. . . (16) and; (d) the induction of IFN-.gamma. production by resting or activated peripheral blood NK cells and T cells (17). **IL-12** is a strong inhibitor of the T cell-dependent synthesis of IgE by IL-4-stimulated peripheral blood mononuclear cells and the IgE. . .

DETDESC:

DETD(213)
Human rIL-12 and Antibodies to **IL-12**

DETDESC:

DETD(214)

Human rIL-12 was produced by cotransfection of COS cells with a 1:1 molar ratio of the two subunit cDNAs of **IL-12** as described by Gubler et al. (13). Crude supernatant fluid from cultures of doubly transfected cells was used as the. . . of rIL-12 in these experiments. Supernatant fluid from cultures of mock transfected COS cells was used as a control. Monoclonal anti-**IL-12** antibody was a 1:1 mixture of two rat monoclonal anti-human **IL-12** antibodies, 4A1 and 20C2, which were isolated and purified as previously described (19). The 4A1 antibody is specific for the **40 kDa** subunit of human **IL-12**, and its isotype is IgG2b. The 20C2 antibody appears to react with the **35 kDa** subunit of **IL-12**, and its isotype is IgG1. These two antibodies were previously found to synergize in blocking **IL-12**-stimulated proliferation of human PHA-activated lymphoblasts.

DETDESC:

DETD(218)

Immunoglobulins . . . cells (50 .mu.g/ml) from those of untreated cells. In preliminary experiments, where the levels of IFN-.gamma. in the supernatants of **IL-12** stimulated cultures were determined at days 2, 4, 6 and 8, we found, in agreement with a previous report (17),. . .

DETDESC:

DETD(221)
IL-12 Suppresses IgE Synthesis

DETDESC:

DETD(222)

As seen in table 17, **IL-12** (60 pM) significantly suppresses the production of IgE and increases the synthesis of IFN-.gamma. by PBMC cultured in the presence of a saturating concentration of IL4 (10 ng/ml). IL-4 significantly but incompletely suppresses the **IL-12**-induced production of IFN-.gamma.; and it totally abolishes the spontaneous production of IFN-.gamma.. The effects of **IL-12** on IgE and IFN-.gamma. production are dose-dependent and they are completely abolished by neutralizing anti-**IL-12** mAbs (FIG. 38). The production of IgG, IgA and IgM in IL-4-stimulated cultures is not significantly affected by **IL-12**. However, IL-4 does not induce the production of IgM, IgA or IgG (with the exception of IgG4) (4). The effect of **Il-12** on pokeweed mitogen (PWM)--induced IgE synthesis was examined. In 3 consecutive experiments, **IL-12** (60 pM) had no significant effect on the PWM-induced synthesis of IgG (1.6.+-.0.1 versus 1.3.+-.0.6 .mu.g/ml; mean.+-.1 SD), IgM (1.2.+-.0.4. . . .

DETDESC:

DETD(223)

As shown in FIG. 38, **IL-12** strongly suppresses the expression of the mature but not of the germ-line C.epsilon. transcript. This indicates that **IL-12** suppresses the synthesis of IgE and possibly inhibits the switching to IgE.

DETDESC:

DETD(224)

IL-12 appears to suppress IgE synthesis by a mechanism which is distinct from that of IFN-.gamma.. The effect of **IL-12** on the synthesis of IgE by umbilical cord blood mononuclear cells (CBMC) costimulated with IL-4 and hydrocortisone was tested. These. . . was added to IL-4 stimulated CMBC for two reasons: (i) HC inhibits the production of IFN-.gamma., even that induced by **IL-12**; (ii) HC strongly increases the IL-4 stimulated synthesis of IgE, even in the absence of IFN-.gamma. production (24). As seen (table 18), **IL-12** markedly inhibits IgE synthesis by neonatal cells cultured in the presence of IL-4 plus hydrocortisone and producing little or nor. . . Moreover, the suppression is unchanged in the presence of a large excess of neutralizing anti-IFN-.gamma. mAb. Thus, it appears that **IL-12** can inhibit IgE synthesis by a mechanism which is independent of IFN-.gamma..

DETDESC:

DETD(225)

As shown above, picomolar concentrations of **IL-12** markedly inhibit the synthesis of IgE by IL-4-stimulated PBMC. The suppression of IgE is observed at the protein and the mRNA levels and it is completely overriden by neutralizing antibodies to **IL-12**. Given that the production of IgE by IL-4-stimulated lymphocytes involves the switching of precursor B cells to IgE rather than the selective expansion and differentiation of IgE committed B cells (4, 25), the results suggest that **IL-12** inhibits the switching to IgE. Consistent with an isotype-specific activity of **IL-12**, no influence is found of this lymphokine on the production of the other classes of Ig by IL-4-stimulated or by PWM-stimulated PBMC. The data do not exclude an effect of **IL-12** on the production of IgG4, the only human isotype

other than IgE which is induced by IL-4 (4). Indeed, the. . .

DETDESC:

DETD(226)

IL-12 induces the production of significant amounts of IFN-.gamma. even in the presence of a high concentration of IL-4, that was. . . as the in vivo differentiation of naive T cells into TH1 type of cells, it is reasonable to assume that **IL-12** may display the same activity even in the presence of IL-4, which may also be produced by non-T cells (26). According to this view, **IL-12** might well pay a pivotal role in determining the outcome of certain immune responses to certain antigens or pathogens that are known to preferentially generate TH1 or TH2 helper cells. The cellular origin of **IL-12** is consistent with a putative role of this lymphokine in the differentiation of naive T cells. Indeed, **IL-12** may be produced not only by Epstein-Barr virus transformed B cells from which it was isolated but also by normal. . .

DETDESC:

DETD(227)

In preliminary experiments using neutralizing antibodies to IFN-.gamma. the **IL-12** mediated suppression of IgE synthesis by adult PBMC was not consistently overcome. These results may be easily explained by (i) the relatively high levels of IFN-.gamma. in **IL-12** containing cultures and (ii) the difficulty in blocking the biological activity of endogenously produced IFN-.gamma.. **IL-12** also suppresses IgE by another mechanism which is IFN-.gamma. independent. The existence of such a mechanism is demonstrated by the ability of **IL-12** to markedly inhibit IgE synthesis by IL-4 and hydrocortisone-costimulated neonatal lymphocytes which do not produce detectable amounts of IFN-.gamma.. It. . . of IgE given that a very large excess of neutralizing anti-IFN-.gamma. antibody failed to increase the IgE response. Whereas both **IL-12** and IFN-.gamma. markedly suppress the accumulation of productive C.epsilon. mRNA in IL-4-stimulated PBMC (>90% suppression), IFN-.gamma., but not **IL-12**, also suppress the expression of germ-like transcript (50-70% inhibition). (5) **IL-12** inhibits IgE synthesis by PBMC costimulated with IL-4 and anti-CD40 mAb, a model where IFN-.gamma. was reported to be inactive. . . mAb 89 (0.5 .mu.g/ml), the production of IgE dropped from 70.+-.28 ng/ml (mean.+-.1SD) to 20.+-.8 ng/ml in the presence of **IL-12** (60 pM) as compared to 79.+-.35 ng/ml in the presence of IFN-.gamma. (100 IU/ml). Taken collectively, the present results demonstrate that, like the interferons, **IL-12** plays an important role not only in protective immunity but also in the regulation of isotype selection.

DETDESC:

DETD(228)

. . .

1 ADDITION	EXP. 2 IgE	IFN-.gamma.	IgE	IFN-.gamma.
--	<0.2	214	<0.2	62
IL-4	57	<1	204	<1
IL-12	<0.2	3364	<0.2	2800
IL-4 + **IL-12**	19	1348	58	810

DETDESC:

DETD(229)
PBMC were cultured for 12 days in the absence or in the presence of IL-4 (10 ng/ml), **IL-12** (60 pM) or both. Shown are the mean values of IgE (ng/ml) and IFN-.gamma. (IU/ml) measured in 4 replicate cultures;. . . 20%. Supernatant fluids from cultures of mock transfected COS cells were inactive when used at the same dilutions as the **IL-12** containing supernatant fluids (not shown).

DETDESC:

DETD(230)

TABLE 18

EFFECT OF **IL-12** ON IgE SYNTHESIS BY NEONATAL LYMPHOCYTES STIMULATED WITH IL-4 AND HYDROCORTISONE

	EXP. 1	EXP. 2	EXP. 3		EXP. 4. . . .		INF-.gamma.	
			IgE	IFN-.gamma.	IgE	IFN-.gamma.	IgE	IFN-.gamma.
--	373	<1	44	<1	20	<1	302	<1
IL-12	6	<1	8	<1	<0.2	17	20	33
IL-12 + Anti- IFN-.gamma.	7	<1	10	<1	<0.2	<1	21	<1
IL-12 + Anti- Lolpl	6	<1	NT	NT	NT	NT	18	128

Umbilical cord blood mononuclear cells were. . .

DETDESC:

DETD(254)

16. . . . F. Sinigaglia, R. Chizonnite, U. Gubler, and A. S. Stern. 1991. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, **IL-12** (cytotoxic lymphocyte maturation factor). J. Immunol. 147:874-882.

DETDESC:

DETD(257)

19. . . . Truitt, F. J. Podlaski, A. G. Wolitzky, P. M. Quinn, P. Nunes, A. S. Stern, and M. K. Gately, 1991. **IL-12**. **Monoclonal antibodies** specific for the **40 kDa** subunit block receptor binding and biologic activity on activated human lymphoblasts. J. Immunol. 147:1548-1556.

TITLE:	**Monoclonal antibodies** to cytotoxic lymphocyte maturation factor		
US PAT NO:	5,780,597 [IMAGE AVAILABLE]	DATE ISSUED:	Jul. 14, 1998
APPL-NO:	08/460,061	DATE FILED:	Jun. 2, 1995
REL-US-DATA:	Division of Ser. No. 205,011, Mar. 2, 1994, abandoned, which is a division of Ser. No. 857,023, Mar. 24, 1992, abandoned, which is a continuation-in-part of Ser. No. 572,284, Aug. 27, 1990, abandoned, which is a continuation-in-part of Ser. No. 520,935, May 9, 1990, abandoned, which is a continuation-in-part of Ser. No. 455,708, Dec. 22, 1989, abandoned.		

US PAT NO: **5,780,597** [IMAGE AVAILABLE] L18: 1 of 1
TITLE: Monoclonal antibodies to cytotoxic lymphocyte maturation factor

ABSTRACT:
The present invention relates to antibodies which bind to a novel cytotoxic lymphocyte maturation factor. When bound to the cytotoxic lymphocyte maturation factor, the antibodies can neutralize bioactivity of the factor.

SUMMARY:

BSUM(5)

The response of a cell to a given cytokine is dependent upon the local **concentration** of the cytokine, the cell type and other cell regulators to which it is concomitantly exposed. The overlapping regulatory actions. . .

DRAWING DESC:

DRWD(40)
A. PBMC were cultured for 12 days in the presence of 10 **ng/ml** of IL-4 and increasing concentrations of IL-12 (0.1 to 100 pM). Shown are the mean.+-.1 SEM of three experiments; *significantly. . .

DRAWING DESC:

DRWD(41)
B. . . . IL-4 and IL-12 (60 pM), with or without anti-IL-12 mAbs (a mixture of antibodies 4A1 and 20C2, each at 10 .mu.g/**ml**).

DRAWING DESC:

DRWD(42)

FIG. . . . accumulation of productive but not germ-like C.epsilon. transcripts. Total RNA was extracted from PBMC cultured for 10 days with 10 **ng/ml** of IL-4, in the absence (lanes 1, 3 and 5) or in the presence of 60 pM IL-12 (lanes 2,. . .

DETDESC:

DETD(8)

Human . . . passage in RPMI 1640 medium supplemented with 5% neat-inactivated (56.degree. C., 30 min.) fetal bovine serum, 2 mM L-glutamine, 100 units/**ml** penicillin, and 100 .mu.g/**ml** streptomycin (all obtained from GIBCO Laboratories, Grand Island, N.Y.).

DETDESC:

DETD(9)

Higher . . . well of three Costar 3596.RTM. microplates (Costar Co., Cambridge, Mass.) received 100 .mu.l of a cell suspension containing five NC-37 cells/**ml**. The medium used for the cloning was a 1:1 mixture of fresh passage medium and filtered, conditioned medium from stock. . .

DETDESC:

DETD(10)

When the number of cells in a given subline exceeded 1.4.times.10.sup.6, one million cells were stimulated to produce CLMF in 1 **ml** cultures containing 3 **ng/ml** phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co., St. Louis, Mo.) and 100 **ng/ml** calcium ionophore A23187 (Sigma). Supernatants were harvested from the cultures after 2 days, dialyzed against about 50 volumes of Dulbecco's. . . overnight with one change of buffer and then for 4 hours against 50 volumes of RPMI 1640 medium with 50 .mu.g/**ml** of gentamicin (both from Gibco) and tested for CLMF by means of the T cell growth factor assay (see below).. . . titers produced by the parent NC-37 cell line. Since cells from these three sublines produced CLMF at similar titers (.gtoreq.800 units/**ml**), culture supernatants derived from the three sublines were pooled for use as starting material for the purification of CLMF.

DETDESC:

DETD(11)

Bulk . . . Cell Production Roller Apparatus Model II, Wheaton Instruments, Millville, N.J.). Cell suspensions were prepared containing 1-1.5.times.10.sup.6 NC-37.89, NC-37.98 or NC-37.102 cells/**ml** of RPMI 1640 supplemented with 1% Nutridoma-SP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 2 mM L-glutamine, 100 units/**ml** penicillin, 100 .mu.g/**ml** streptomycin, 10 **ng/ml** PMA and 20-25 **ng/ml** calcium ionophore A23187. Two hundred fifty to three hundred fifty **ml** aliquots of the cell suspensions were added to Falcon 3027 tissue culture roller bottles (Becton Dickinson, Lincoln Park, N.J.) which. . . concentrations of 1 mM and 0.1 mM, respectively, to retard proteolytic degradation. The supernatants were stored at 4.degree. C. until **concentration**.

DETDESC:

DETD(13)

Culture . . . method. Blood from normal volunteer donors was drawn into syringes containing sufficient sterile preservative-free heparin (Sigma) to give a final **concentration** of approximately 5 units/**ml**. The blood was diluted 1:1 with Hanks' balanced salt solution (HBSS) without calcium or magnesium (GIBCO). The diluted blood was then layered over 15 **ml** aliquots of Ficoll/sodium diatrizoate solution (Lymphocyte Separation Medium, Organon Teknika Corp., Durham, N.C.) in 50 **ml** Falcon 2098 centrifuge tubes. The tubes were centrifuged for 30 minutes at room temperature at 500.times.g. Following centrifugation, the cells. . . by mixing with .gtoreq.2 volumes of HBSS without calcium or magnesium. The resulting cell suspension was then layered over 15 **ml** aliquots of 20% sucrose (Fisher) in RPMI 1640 medium with 1% human AB serum (Irvine Scientific, Santa Ana, Calif.) in. . .

DETDESC:

DETD(14)

The . . . composed of a 1:1 mixture of RPMI 1640and Dulbecco's modified Eagle's medium, supplemented with 0.1 mM nonessential amino acids, 60 .mu.g/**ml** arginine HCl, 10 mM HEPES buffer, 2 mM L-glutamine, 100 units/**ml** penicillin, 100 .mu.g/**ml** streptomycin (all available from GIBCO), 5.times.10.sup.-5 M 2-mercaptoethanol (Fisher Scientific, Fair Lawn, N.J.), 1 mg/**ml** dextrose (Fisher), and 5% human

AB serum (Irvine Scientific, Santa Ana, Calif.). These cells were incubated in 24-well tissue culture plates (Costar, Cambridge, Mass.) in 1 **ml** cultures (7.5.times.10.sup.5 cells/culture) to which 10.sup.-4 M hydrocortisone sodium succinate (Sigma) was added to minimize endogenous cytokine production. Some cultures also received human rIL-2 (supplied by Hoffmann-La Roche) at a final **concentration** of 5 units/**ml** and/or supernatants to be assayed for CLMF activity. All cultures were incubated for 3-4 days at 37.degree. C. in a. . .

DETDESC:

DETD(15)

At . . . this incubation, the contents of each culture were harvested, and the cells were pelleted by centrifugation and resuspended in 0.5 **ml** of fresh TCM. One tenth **ml** aliquots of these cell suspensions were mixed with 0.1 **ml** aliquots of .sup.51 Cr-labelled K562 or Raji cells (both cell lines obtained from the ATCC) and tested for their lytic. . .

DETDESC:

DETD(16)

LAK . . . above were added to the wells of Costar 3596 microplates (5.times.10.sup.4 cells/well). Some of the wells also received rIL-2 (5 units/**ml** final **concentration**) and/or purified CLMF or immunodepleted CLMF-containing solutions. All cultures contained 10.sup.-4 M hydrocortisone sodium succinate (Sigma) and were brought to a total volume of 0.1 **ml** by addition of TCM with 5% human AB serum. The cultures were incubated for 3 days at 37.degree. C., after which 0.1 **ml** of .sup.51 Cr-labelled K562 cells (5.times.10.sup.4 cells/**ml** in TCM with 5% human AB serum) were added to each well. The cultures were then incubated overnight at 37.degree.. . .

DETDESC:

DETD(18)

Methods . . . recovered from the interface between the 45% and 58% Percoll layers were used as responder lymphocytes in mixed lymphocyte-tumor cultures (**MLTC**). CTL were generated in **MLTC** in 24-well tissue culture plates (Costar #3424) by incubation of Percoll gradient-derived high density lymphocytes (7.5.times.10.sup.5 /culture) together with 1.times.10.sup.5. . . melanoma cells (ATCC, Rockville, Md.) or with 5.times.10.sup.4 gamma-irradiated HT144 melanoma cells in TCM with 5% human AB serum (1.2 **ml**/culture). For uv-irradiation, HT144 cells were suspended at a density of 1-1.5.times.10.sup.6 cells/**ml** in Hanks' balanced salt solution without phenol red (GIBCO) containing 1% human AB serum. One **ml** aliquots of the cell suspension were added to 35.times.10 mm plastic tissue culture dishes (Falcon #3001), and the cells were. . . MINERALIGHT.RTM. lamp, Ultra-violet Products, Inc., San Gabriel, Calif.). For gamma irradiation, HT144 cells were suspended at a density of 1-5.times.10.sup.6 cells/**ml** in TCM with 5% human AB serum and irradiated (10,000 rad) by use of a cesium source irradiator (model 143,. . . were centrifuged and resuspended in TCM with 5% human AB serum at the desired cell density for addition to the **MLTC**. In addition to lymphocytes and melanoma cells, some **MLTC** received human rIL-2 and/or purified human CLMF at the concentrations indicated in the table. Hydrocortisone sodium succinate (Sigma) was added to the **MLTC** at a final **concentration** of 10.sup.-4 M (cultures containing uv-irradiated melanoma cells) or 10.sup.-5 M (cultures containing gamma-irradiated melanoma cells) to supress endogenous cytokine. . . air for 6 days. At the end of this time, lymphocytes from replicase cultures were pooled, centrifuged,

resuspended in 1.2 **ml** TCM containing 5% human AB serum, and tested for their ability to lyse HT144 melanoma cells, and, as a specificity. . .

DETDESC:

DETD(19)

Melanoma . . . et al. (ibid.) for quantitating lysis of glioma target cells. For assaying the lysis of .sup.51 Cr-labeled K562 cells, 0.1 **ml** aliquots of lymphocyte suspensions were mixed with 25 .mu.l aliquots of .sup.51 Cr-labeled K562 (2.times.10.sup.5 cells/**ml** in TCM with 5% human AB serum) in the wells of Costar 3696 "half-area" microtest plates. After overnight incubation at. . .

DETDESC:

DETD(21)

The . . . were isolated by centrifugation over discontinuous Ficoll and sucrose gradients as described above for the LCI assay. The PBMC (5.times.10.sup.5 cells/**ml**) were cultured at 37.degree. C. in TCM containing 0.1% phytohemagglutinin-P (PHA-P) (Difco Laboratories, Detroit, Mich.). After 3 days, the cultures were split 1:1 with fresh TCM, and human rIL-2 was added to each culture to give a final **concentration** of 50 units/**ml**. The cultures were then incubated for an additional 1 to 2 days, at which time the cells were harvested, washed, and resuspended in TCM at 4.times.10.sup.5 cells/**ml**. To this cell suspension was added heat-inactivated goat anti-human rIL-2 antiserum (final dilution: 1/200) to block any potential IL-2-induced cell. . . antiserum, which was provided by R. Chizzonite, Molecular Genetics Department, Hoffmann-La Roche, was shown to cause 50% neutralization of 2 units/**ml** rIL-2 at a serum dilution of 1/20,000. An equally functional anti-human rIL2 antibody can be obtained from the Genzyme Co.,. . .

DETDESC:

DETD(22)

Fifty . . . a humidified atmosphere of 5% CO.sub.2 in air, and 50 .mu.l of .sup.3 H-thymidine (New England Nuclear, Boston, Mass.), 10 .mu.Ci/**ml** in TCM, were then added to each well. The cultures were further incubated overnight. Subsequently, the culture contents were harvested. . .

DETDESC:

DETD(23)

In . . . NC-37 cells was used as a standard. Several dilutions of this preparation, which was assigned an arbitrary titer of 2000 units/**ml**, were included in each TGF or LAK induction assay. The results obtained for the standard preparation were used to construct a dose-response curve from which could be interpolated units/**ml** of activity in each unknown sample at the dilution tested. Multiplication of this value by the dilution factor yielded the activity of the original sample expressed in units/**ml**.

DETDESC:

DETD(24)

For . . . mixtures were incubated for 30 min. at 37.degree. C. and 25 .mu.l aliquots of a suspension of PHA-activated lymphoblasts

(8.times.10.sup.5 /**ml** in TCM plus 1:100 anti-rIL-2) were then added to each well. The cultures were further incubated, pulsed with .sup.3 H-thymidine,. . .

DETDESC:

DETD(26)

Purified . . . sucrose gradients as described above and were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/**ml** penicillin, 100 .mu.g/**ml** streptomycin, and 2 mM L-glutamine. The PBMC were incubated overnight at 37.degree. C. in 1 **ml** cultures (5.times.10.sup.6 cells/culture) together with rIL-2 and/or purified CLMF at various concentrations. After 18-20 hours, the contents of the cultures. . .

DETDESC:

DETD(28)
Concentration of Cell Supernatant Solutions

DETDESC:

DETD(31)

The concentrated supernatant solution was applied at a flow rate of 120 **ml**/hr to a NuGel P-SP (Separation Industries, Metuchen, N.J.) column (5.times.5 cm), equilibrated in 10 mM MES, pH 6.0. The column was washed until baseline absorbance monitoring at 280 nm was obtained. Absorbed proteins were then eluted with a 500 **ml** salt gradient from 0 to 0.5M NaCl/10 mM MES, pH 6.0 at a flow rate of 2 **ml**/min. Aliquots of fractions were assayed for TGF activity. Fractions containing TGF activity were pooled and dialyzed (Spectra/Por 7, Fisher Scientific). . .

DETDESC:

DETD(33)

The . . . 10 min at 4.degree. C. and the precipitate discarded. The supernatant solution was applied at a flow rate of 20 **ml**/hr to a Blue B-Agarose (Amicon, Danvers, Mass.) column (2.5.times.10 cm) equilibrated in 20 mM Tris/HCl, pH 7.5. The column was. . . this same buffer until baseline absorbance monitoring at 280 nm was obtained. Absorbed proteins were then eluted with a 500 **ml** salt gradient from 0 to 0.5M NaCl/20 mM Tris/HCl, pH 7.5 at a flow rate of 15 **ml**/hr. Aliquots of fractions were assayed for TGF activity. Fractions containing TGF activity were pooled and dialyzed (Spectra/Por 7, Fisher Scientific). . .

DETDESC:

DETD(35)

The . . . a 0.45 .mu.m cellulose acetate filter (Nalgene Co., Rochester, N.Y.) and the filtrate applied at a flow rate of 60 **ml**/hr to a Mono Q HR 5/5 (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) column (5.times.50 mm) equilibrated in 20 mM Tris/HCl,. . . 1 hr linear salt gradient from 0 to 0.25M NaCl/20 mM Tris/HCl, pH 7.5 at a flow rate of 60 **ml**/hr. Aliquots of fractions were assayed for TGF activity and protein purity was assessed without reduction by SDS-PAGE [Laemmli, U.K. (1970). . .

DETDESC:

DETD(54)

Purification . . . by a modification of the Iodogen method (Pierce Chemical Co.). Iodogen (Pierce Chemical Co.) was dissolved in chloroform at a **concentration** of 0.5 mg/**ml** and 0.1 **ml** aliquots were added to 12.times.75 borosilicate glass tubes. The chloroform was evaporated under a stream of nitrogen and the Iodogen. . . temperature (RT) under vacuum. For radiolabeling, 0.5-1.0 mCi .sup.125 I-Na (Amersham) was added to an Iodogen coated tube containing 0.50 **ml** of Tris-Iodination Buffer (25 mM Tris-HCl pH 7.5, 0.4M NaCl, 1 mM EDTA) and incubated for 4 minutes at RT. The activated .sup.125 I solution was transferred to a 1.5 **ml** tube containing 0.05-0.1 **ml** CLMF (approximately 5 .mu.g in 0.125M NaCl, 20 mM Tris-HCl pH 7.5) and the reaction was further incubated for 8 minutes at RT. At the end of the incubation, 0.05 **ml** of Iodogen Stop Buffer (10 mg/**ml** tyrosine, 10% glycerol in Dulbecco's phosphate buffered saline (PBS) pH 7.4) was added and reacted for 30 seconds. The mixture was then diluted with 1.0 **ml** Tris-Iodination Buffer and applied to a BioRad BioGel P10DG BioRad Laboratories) desalting column for chromatography. The column was eluted with Tris-Iodination Buffer and fractions (1 **ml**) containing the peak amounts of labelled protein were combined and diluted to 1.times.10.sup.8 cpm/**ml** with 0.25% gelatin in Tri iodination buffer. The TCA precipitable radioactivity (10% trichloroacetic acid final **concentration**) was typically in excess of 95% of the total radioactivity. The radiospecific activity ranged from 6000 cpm/fmol to 10,000 cpm/fmol.

DETDESC:

DETD(55)

Immunodepletion . . . immunodeplete CLMF as follows: Goat anti-rat IgG-agarose beads (Sigma Chemical Co., St. Louis, Mo.) were washed three times with 10 **ml** of PBS (Gibco) supplemented with 1% bovine serum albumin (BSA) (Sigma) (PBS/BSA solution). After washing, the beads were resuspended in PBS/BSA at a final **concentration** of 50% vol/vol. Aliquots (0.2 **ml**) of the bead suspension were added to 1.5 **ml** Eppendorf tubes, together with the indicated amounts of monoclonal antibodies or hybridoma supernatant solutions. The volume of each mixture was brought to 1.4 **ml** by the addition of hybridoma maintenance medium [Iscove's modified Dulbecco's medium (IMDM) with 0.1% fetal bovine serum (FBS), 10% Nutridoma-SP. . . 5), and the supernatants were discarded. The beads were again washed three times with PBS/BSA and then resuspended in 1 **ml** of tissue culture medium (TCM) containing 5% human AB serum and the indicated **concentration** of purified human CLMF. The tubes were subsequently incubated overnight at 4.degree. C. on the mixer. Following this, the beads. . .

DETDESC:

DETD(56)

Immunoprecipitation Assay. For the immunoprecipitation reaction, 0.05 to 0.5 **ml** of hybridoma supernatant, diluted antisera or purified IgG was added to a 1.5 **ml** microfuge tube containing 0.1 **ml** of a 50% suspension of goat-anti-rat IgG coupled to agarose (Sigma Chemical Co.). The assay volume was brought up to 0.5 **ml** with RIPA Buffer (50 mM NaPO.sub.4 pH 7.5, 150 mM NaCl, 1% Triton-X 100, 1% Deoxycholic acid, 0.1% SDS, 1%. . . 2 hours at RT. The beads were pelleted by centrifugation for 1 minute at 12,000.times.g and then resuspended in 1 **ml** RIPA Buffer containing .sup.125 I CLMF (1.times.10.sup.5 cpm). The mixture was then incubated on a rotating mixer for 16 hours. . .

DETDESC:

DETD(57)

CLMF . . . IgG or antisera to inhibit the binding of .sup.125 I-CLMF to PHA-activated human T lymphoblasts was measured as follows: 0.1 **ml** aliquots of serial dilutions of culture supernatants, purified IgG or antisera were mixed with 0.025 **ml** aliquots of Binding Buffer (RPMI-1640, 5% FBS, 25 mM HEPES pH 7.4) containing .sup.125 I-CLMF (1.times.10.sup.5 cpm). The mixture was incubated on an orbital shaker for 1 hour at RT, then 0.025 **ml** of activated blasts (5.times.10.sup.7 cells/**ml**) was added to each tube. The mixture was further incubated for 1 hour at RT. Non-specific binding was determined by. . . duplicate or triplicate. Cell bound radioactivity was separated from free .sup.125 I-CLMF by centrifugation of the assay contents through 0.1 **ml** of an oil mixture (1:2 mixture of Thomas Silicone Fluid 6428-R15: A. H. Thomas, and Silicone Oil AR 200: Gallard-Schlessinger). . . .

DETDESC:

DETD(58)

SDS . . . was washed with wash buffer and the bound antibody visualized by incubation for 30 minutes at RT with 4-chloro-1-napthol (0.5 mg/**ml** in 0.15% H.sub.2 O.sub.2, 0.5M NaCl, 50 mM Tris-HCl, pH 7.5). The reaction was stopped by extensive washing with distilled. . . .

DETDESC:

DETD(63)

The . . . growth factor (TGF) activity was determined and the active fractions were pooled and dialyzed in order to reduce the salt **concentration** of the preparation by 50-fold. This material, after centrifugation to remove particulates, was applied to a Blue-B-Agarose column. Protein was. . . 2). Peak TGF activity was determined and the active fractions were pooled and dialyzed in order to reduce the salt **concentration** of the preparation by 100-fold. This material, after filtration, was applied to a Mono Q column. Protein was eluted with. . .

DETDESC:

DETD(64)

Therefore, . . . 8M urea and pumped onto a Vydac diphenyl column using an enrichment technique. The column was then washed with 5 **ml** of 0.1% trifluoroacetic acid. Elution of the proteins was accomplished with a gradient of 0-70% acetonitrile over 7 hrs in. . .

DETDESC:

DETD(69)

. . .

Step	Pooled Volume (**ml**)	Activity (U/**ml**)	Total Pooled Units (U)	Protein (mg/**ml**)	Total Protein (mg)	Specific Activity (U/mg)

Pooled Cell
60,000
2.58 .times. 10.sup.3
1.6 .times. 10.sup.8
ND ND ND
Supernatants. . .

DETDESC:

DETD(77)

The . . . 8). Peak TGF activity was determined and the active fractions were pooled and dialyzed in order to reduce the salt **concentration** of the preparation by 50-fold. This material, after centrifugation to remove particulates, was applied to a Blue-B-Agarose column. Protein was. . . 9). Peak TGF activity was determined and the active fractions were pooled and dialyzed in order to reduce the salt **concentration** of the preparation by 100-fold. This material, after filtration, was applied to a Mono Q column. Protein was eluted with. . .

DETDESC:

DETD(78)

Fractions . . . 8M urea and pumped onto a Vydac diphenyl column using an enrichment technique. The column was then washed with 5 **ml** of 0.1% trifluoroacetic acid. Elution of the proteins was accomplished with a gradient of 0-70% acetonitrile over 7 hrs in. . .

DETDESC:

DETD(105)

Fraction . . . sample was pumped onto a Vydac C-18 column using an enrichment technique and the column was then washed with 5 **ml** of 0.1% trifluoroacetic acid. Elution of the proteins was accomplished with a gradient of 0-70% acetonitrile over 5 hrs in. . .

DETDESC:

DETD(115)

Mono Q fractions 36 and 37 from Example 1 were pooled (approximately 100 pmol/1.7 **ml**) and the volume (less 30 .mu.l--see Example 6) reduced to 200 .mu.l under a stream of helium. One hundred microliters. . . 8.5/6M guanidine-HCl. The volume was reduced to 200 .mu.l under a stream of helium, and 4 .mu.l of dithiothreitol (50 mg/**ml**) was added. The mixture was incubated at 37.degree. C. for 4 hrs. After reductive cleavage of the disulfide bonds, [.sup.14. . .

DETDESC:

DETD(129)
1. 100 **ml** Sepharose CL-6B was washed three times with 100 **ml** H.sub.2 O.

DETDESC:

DETD(130)
2. 100 **ml** of 1% sodium meta-periodate in H.sub.2 O was added to the resin and the suspension shaken at room temperature for. . .

DETDESC:

DETD(133)
1. 9 **ml** of the activated agarose (described above) was suspended in 7 **ml** of 7B2 (@3.9 mg/**ml**) in phosphate buffered saline, pH 7.4.

DETDESC:

DETD(135)
3. The gel suspension was filtered and added to 7 **ml** of 1.0M ethanolamine, pH 7.0 containing 50.2 mg of cyanoborohydride.

DETDESC:

DETD(136)

One millimeter of the above described resin (@2.6 mg IgG/**ml** gel) was packed in a column and washed extensively with phosphate buffered saline. Fractions from the Mono Q chromatography containing. . . TGF activity) and dialyzed extensively against PBS. This preparation was applied to the 7B2-Sepharose column at a rate of 5 **ml**/hr at room temperature. The column was washed with phosphate buffered saline (pH 7.4) until baseline absorbance monitoring at 280 nm. . . .

DETDESC:

DETD(144)

The . . . and in an overnight NK cell activation assay. In the LCI assay, purified CLMF at concentrations as high as 800 units/**ml** had little activity in the absence of IL-2 (Table 9). However, CLMF synergized with low concentrations of human rIL-2 in. . . either cytokine alone (Table 9). In the presence of rIL-2, purified CLMF was active at concentrations as low as 3 units/**ml**.

DETDESC:

DETD(145)

In . . . NK cells in an overnight assay (Table 10). In this assay, CLMF was active at concentrations as low as 1.6 units/**ml**. When CLMF was tested in combination with human rIL-2, the two cytokines together had, at best, additive effects in enhancing. . .

DETDESC:

DETD(146)

In . . . increased the specific allogeneic CTL response to weakly immunogenic, gamma-irradiated HT144 melanoma cells (Table 11). In combination with a low **concentration** of rIL-2, CLMF also facilitated specific allogeneic human CTL responses to uv-irradiated HT144 melanoma cells, which did not elicit any. . .

DETDESC:

DETD(148)
. . .
of
Human PHA-Activated Lymphoblasts
Cytokine Added: .sup.3 H-Thymidine Incorporated by
Human CLMF.sup.C
Human rIL-2
PHA-Activated Lymphoblasts
Expt.
(u/**ml**) (u/**ml**) (mean cpm + 1 S.E.M.)

1.sup.a

0	0	10,607 .+-. 596
500	0	70,058 .+-. 1,630

. . .

DETDESC:

DETD(149)

. . .

Killer (LAK) Cells in 4-Day Cultures

Cytokine Added: Human CLMF.sup.b (u/ml)	Human rIL-2 (u/ml)	% Specific .sup.51 Cr Release.sup.a from: K562	Raji
0	0	3 .+-. 1.7	-1 .+-. 0.5
800	0	7 .+-. 0.3	1 .+-.. . .

DETDESC:

DETD(150)

. . .

Cytokine Added: Human CLMF.sup.b (u/ml)	Human rIL-2 (u/ml)	% Specific .sup.51 Cr Release.sup.a from Raji Cells at Effector/Target Ratio =: 20/1	5/1
0	0	10 .+-. 0.6	5 .+-. 0.4
40	0	31 .+-. 0.4	14 .+-.. . .

DETDESC:

DETD(151)

. . .

Lymphocytes (Percoll fraction.sup.b)	Hydrocortisone (M)	Melanoma Cells.sup.c	rIL-2 (u/ml)	CLMF (u/ml)	Expt. 1 HT144	Expt. 1 K562	Expt. 2 HT144	Expt. 2 K562
4	10.sup.-4				6 .+-. 3	-4 .+-. 1	-2 .+-.. . .	47 .+-. 1

.sup.a In both experiments the contents of duplicate cultures were pooled

washed, resuspended in 1.2 **ml** TCM, and assayed for lytic activity undiluted and at 1:5 dilution. In experiment 1, the data shown were . . .

DETDESC:

DETD(155)

NC . . . portion of the culture was continued for 3 days, at which point the bioassay titer for CLMF activity read 2200 units/**ml**, indicating that the cells harvested for isolation of RNA had indeed produced the CLMF activity. Total RNA was isolated from. . .

DETDESC:

DETD(164)

A total of 3.times.10.sup.5 clones from the amplified library were screened on 6 duplicate filters under the following conditions: 50 **ml** of 5.times.SSC/10.times. Denhardts/100 .mu.g/**ml** denatured calf thymus DNA/20% formamide/0.1% SDS/1.5.times.10.sup.6 cpm of labelled 54 mer at 37.degree. C. for 16 hours. The filters were. . .

DETDESC:

DETD(174)

A . . . the amplified 16 h library were screened on 40 duplicate filters with the above probe under the following conditions: 400 **ml** of 5X SSC/20% formamide/10X Denhardts/100 .mu.g/**ml** denatured calf thymus DNA/0.1% SDS/3.8.times.10.sup.7 cpm labelled probe at 37.degree. C. overnight. The filters were subsequently washed in 2X SSC. . .

DETDESC:

DETD(184)

Supernatant . . . by the culture fluid from doubly transfected COS cells to the amount of proliferation induced by purified NC-37-derived CLMF, the **concentration** of CLMF activity in the culture fluid was estimated to be 374 units/**ml**. Assuming a specific activity of 8.times.10.sup.7 units/mg CLMF protein, this result suggests that the fluid from cultures of doubly transfected COS cells contained approximately 4.7 **ng/ml** of recombinant CLMF.

DETDESC:

DETD(185)

. . .

Growth Factor Activity of Recombinant CLMF
Expressed in COS cells

Cytokine added:	**Concentration**:	.sup.3 H-Thymidine Incorporated by PHA-Activated Lymphoblasts (mean cpm .+-. 1 S.E.M.)
None		14,587 .+-. 343
Natural CLMF*	200 units/**ml**	79,848 .+-. 854
Natural CLMF	40 units/**ml**	59,093 .+-. 2029
Natural CLMF	8 units/**ml**	39,180 .+-. 545
Natural CLMF	1.6 units/**ml**	

25,996 .+-. 763

Supernatant fluid from cultures of COS cells transfected with:
35 kDa CLMF subunit cDNA
1/5. . .

DETDESC:

DETD(188)

Lewis . . . NSO cells) with 35% polyethylene glycol (PEG 4000, E. Merck). The fused cells were plated at a density of 5.times.10.sup.4 cells/well/**ml** in 48 well plates in IMDM supplemented with 15% FBS, glutamine (2 mM), beta-mercaptoethanol (0.1 mM), gentamycin (50 **ug/ml**), HEPES (10 mM) and 15% P388D1 cell supernatant. Hybridoma supernatants were screened for specific CLMF antibodies in 4 assays: 1). . .

DETDESC:

DETD(189)

Isolation . . . Serum isolated at the 3rd bleed from the rat immunized with partially purified CLMF (Table 13) neutralized CLMF bioactivity (5 units/**ml**) as determined in the TGF assay (FIG. 27). This neutralization could be blocked by adding excess CLMF (200 units/**ml**) demonstrating that the neutralization by the antiserum was specific for CLMF (FIG. 27). Normal rat serum did not neutralize CLMF. . .

DETDESC:

DETD(191)

After . . . Immunodepletion of CLMF activity by immobilized anti-CLMF antibodies occurs in a dose dependent manner (FIG. 29). Aliquots (0.4 and 0.1 **ml**) of hybridoma supernatant solution will completely deplete 50 and 200 units/**ml** of CLMF activity from the culture medium. 0.025 **ml** of supernatant solution will completely deplete 50 units/**ml** but only approximately 50% of 200 units/**ml**. 0.0062 **ml** of hybridoma supernatant shows even less depletion of 50 and 200 units/**ml** of CLMF. An aliquot (0.4 **ml**) of an anti-IL-1 receptor antibody supernatant solution shows no immunodepletion of CLMF bioactivity.

DETDESC:

DETD(196)

Three . . . 2A3 and 7B2 inhibit in a dose dependent manner .sup.125 I-CLMF binding to the lymphoblasts with IC.sub.50 concentrations of 0.7 .mu.g/**ml** 7 .mu.g/**ml** and 9.5 .mu.g/**ml**, respectively (FIG. 36). Antibodies 6A3 and 8E3 do not block .sup.125 I-CLMF binding at concentrations of 100 .mu.g/**ml** (FIG. 36). These data demonstrated that the original classification of each antibody as either inhibitory or non-inhibitory was correct.

DETDESC:

DETD(197)

Direct . . . the TGF assay (Table 15). Two inhibitory antibodies, 4A1 and 7B2, demonstrated a dose dependent neutralization of CLMF bioactivity (40 units/**ml**) from 0.03 to 100 .mu.g/**ml**, with IC.sub.50

concentrations of approximately 1 .mu.g/**ml** and 80 .mu.g/**ml**, respectively. These data confirmed that antibodies inhibiting .sup.125 I-CLMF binding to the CLMF receptor would also neutralize CLMF bioactivity.

DETDESC:

DETD(199)

. . .

by the TGF assay: A antibody was considered neutralizing if it would block more than 50% proliferation at 200 .mu.g/**ml**. The results are presented as positive (+) or negative (-).
.sup.4 ND: Not Determined

DETDESC:

DETD(200)

. . .

	Antibody.sup.b	Incorporation	% Neutralization.sup.c
none	none	9923 .+-. 439	
CLMF	none	25752 .+-. 592	
CLMF	4A1		
100	.mu.g/**ml**	12965 .+-. 938	81
20		12215 .+-. 663	86
4		12985 .+-. 269	81
.8		19932 .+-. 1016	37
.16		22379 .+-. 410	21
.03		25405 .+-. 1093	2
CLMF	7B2		
200	.mu.g/**ml**	10763 .+-. 878	96
100		15083 .+-. 406	67
20		23690 .+-. 1228	13
4		25849 .+-. 1408	0
CLMF	Control		
200	.mu.g/**ml**	27654 .+-. 1086	0
100		22221 .+-. 381	22
20		27335 .+-. 620	0

.sup.a Purified CLMF was used in the TGF assay at a **concentration** of 40 units/**ml**.
.sup.b Purified antibodies were added at the concentrations indicated in the table.
.sup.c Reduction of .sup.3 Hthymidine. . . .

DETDESC:

DETD(204)

Serum . . . with the synthetic peptide in a direct ELISA assay. The synthetic, free peptide was coated on microtiter plates at 4 **ng/ml** and 20 **ng/ml**, and the plates were washed and blocked with bovine serum albumin. Serum samples were tested at various dilutions (Table 16),. . .

DETDESC:

DETD(205)

A . . . for reactivity with 75 kDa CLMF and with the 35 kDa CLMF subunit (FIG. 37). Partially purified CLMF (approximately 120 .mu.g/**ml**) was run on SDS-PAGE, transferred to nitrocellulose, and treated with a 1:500 dilution of the rabbit anti-CLMF peptide antiserum. Antibody. . .

DETDESC:

DETD(207)

. . .
SUBUNIT OF CLMF
Serum Source
Free A.sub.490
Rabbit
Bleed
Peptide
Serum Dilutions (fold)
No. No.
(**ng/ml**)
1 .times. 10.sup.4
2.5 .times. 10.sup.4
6.2 .times. 10.sup.4
1.6 .times. 10.sup.5
3.9 .times. 10.sup.5. . .

DETDESC:

DETD(212)

Human . . . Island, N.Y.); anti-IFN-.gamma. neutralizing mAb was purchased from Genzyme, (No. 1598-00 Boston, Mass.). In preliminary titration experiments, this antibody (25 .mu.g/**ml**) completely neutralized the suppressive activity of 500 IU/**ml** of IFN-.gamma. on the IL-4-stimulated synthesis of IgE by PBMC. IgE (**ng/ml**) in IL-4-stimulated cultures was 30.+-.4 as compared to 9.8.+-.2 in the presence of IFN-.gamma. (500 IU/**ml**) and to 31.7.+-.3.8 in the presence of both IFN-.gamma. and anti-IFN-.gamma. mAb. Anti-Lolpl mAb is a mouse IgG1 antibody directed. . .

DETDESC:

DETD(216)

Cells . . . HB101 culture medium (Hana Biologics Inc., Alemeda, Calif.) supplemented with 5% fetal calf serum (Flow Labs, McLean, Va.) penicillin (100 U/**ml**), streptomycin (100 .mu.g/**ml**, L glutamine (2 mM) (Gibco Laboratories), sodium pyruvate (10 mM) and Hepes (10 mM). Cells (2.times.10.sup.5 in 0.2 **ml**) were cultured in four replicates or more in round-bottomed 96-well tissue culture plates (Linbro) for 12 days in a humidified. . . of 5% CO.sub.2 and 95% air. For the induction of IgE synthesis, cultures were supplemented with IL-4 at the

final **concentration** of 10 **ng/ml**; this **concentration** was found to be optimal for the induction of IgE synthesis and the suppression of IFN-.gamma. production in mixed lymphocyte. . .

DETDESC:

DETD(218)

Immunoglobulins . . . as described (20,21); IFN-.gamma. was measured by a commercially available RIA (Centocor Co., Malvern, Pa.) with a sensitivity of 1 IU/**ml**. The net synthesis of Igs and of IFN-.gamma. was determined by subtracting the values measured in the culture supernatants of cycloheximide-treated cells (50 .mu.g/**ml**) from those of untreated cells. In preliminary experiments, where the levels of IFN-.gamma. in the supernatants of IL-12 stimulated cultures. . .

DETDESC:

DETD(220)

Northern . . . at 80.degree. C. under vacuum, prehybridized in 50% formamide--5 X Denhardt's--5X SSC--10 mM EDTA--50 mM sodium phosphate pH 6.8-0.1% SDS--250 .mu.g/**ml** salmon sperm DNA and incubated overnight at 42.degree. C. with .sup.32 P labelled cDNA probe in the same buffer. The. . .

DETDESC:

DETD(222)

As . . . suppresses the production of IgE and increases the synthesis of IFN-.gamma. by PBMC cultured in the presence of a saturating **concentration** of IL4 (10 **ng/ml**). IL-4 significantly but incompletely suppresses the IL-12-induced production of IFN-.gamma.; and it totally abolishes the spontaneous production of IFN-.gamma.. The. . . In 3 consecutive experiments, IL-12 (60 pM) had no significant effect on the PWM-induced synthesis of IgG (1.6.+-.0.1 versus 1.3.+-.0.6 .mu.g/**ml**; mean.+-.1 SD), IgM (1.2.+-.0.4 versus 1.1.+-.0.6 .mu.g/**ml**) and of IgA (1.9.+-.0.6 versus 2.1.+-.0.7 .mu.g/**ml**).

DETDESC:

DETD(226)

IL-12 induces the production of significant amounts of IFN-.gamma. even in the presence of a high **concentration** of IL-4, that was shown to completely suppress IFN-.gamma. production and to induce IgE synthesis in mixed lymphocyte cultures (6,7).. . .

DETDESC:

DETD(227)

In . . . reported to be inactive (27). In three such experiments where PBMC were cultured with IL-4 and anti-CD40 mAb 89 (0.5 .mu.g/**ml**), the production of IgE dropped from 70.+-.28 **ng/ml** (mean.+-.1SD) to 20.+-.8 **ng/ml** in the presence of IL-12 (60 pM) as compared to 79.+-.35 **ng/ml** in the presence of IFN-.gamma. (100 IU/**ml**). Taken collectively, the present results demonstrate that, like the interferons, IL-12 plays an important role not only in protective immunity. . .

DETDESC:

DETD(229)

PBMC were cultured for 12 days in the absence or in the presence of IL-4 (10 **ng/ml**), IL-12 (60 pM) or both. Shown are the mean values of IgE (**ng/ml**) and IFN-.gamma. (IU/**ml**) measured in 4 replicate cultures; the variation between the replicates was below 20%. Supernatant fluids from cultures of mock transfected. . .

DETDESC:

DETD(230)

. . .
for 12 days in the presence of IL4 and 10 .mu.M hydrocortisone. IL12 (60 pM), antiIFN-.gamma mAb (1000 neutralizing U/**ml**) or the isotypematched control (antiLolpl) mA (50 .mu.g/**ml**) were added at the initiation of the culture. IgE (**ng/ml**) an IFN.gamma. (IU/**ml**) were measured at day 12 and day 6 respectively. Shown are the mean values of quadruplicate cultures; the variation. . .

DETDESC:

DETD(234)

3.0 mg/**ml** CLMF is dissolved in phosphate-buffered saline pH 7 (PBS pH 7) q.s. 1 **ml**. To this solution may be added one of the following: 0.2 mg/**ml** Polysorbate 80; or 5.0 mg human serum albumin; or 10.0 mg/**ml** benzyl alcohol. Alternatively, a formulation including 3.0 mg/**ml** CLMF; 25 mg/**ml** mannitol; and Tris buffer pH 7 q.s. 1 **ml** may be used.

DETDESC:

DETD(235)

A preferred formulation which is a lyophilized powder for injection includes 3.0 mg/**ml** CLMF, 3.0 mg/**ml** trehalose, and PBS pH 7 q.s. 1 **ml**.

TITLE: Monoclonal antibodies to cytotoxic lymphocyte maturation factor
US PAT NO: **5,780,597** [IMAGE AVAILABLE] DATE ISSUED: Jul. 14, 1998
APPL-NO: 08/460,061 DATE FILED: Jun. 2, 1995
REL-US-DATA: Division of Ser. No. 205,011, Mar. 2, 1994, abandoned, which is a division of Ser. No. 857,023, Mar. 24, 1992, abandoned, which is a continuation-in-part of Ser. No. 572,284, Aug. 27, 1990, abandoned, which is a continuation-in-part of Ser. No. 520,935, May 9, 1990, abandoned, which is a continuation-in-part of Ser. No. 455,708, Dec. 22, 1989, abandoned.

US PAT NO: 5,780,597 [IMAGE AVAILABLE] L3: 7 of 44
TITLE: Monoclonal antibodies to cytotoxic lymphocyte maturation factor

ABSTRACT:

The present invention relates to antibodies which bind to a novel cytotoxic lymphocyte maturation factor. When bound to the cytotoxic lymphocyte maturation factor, the antibodies can neutralize bioactivity of the factor.

DRAWING DESC:

DRWD(41)

B. . . . in the presence of IL-4 and IL-12 (60 pM), with or without anti-IL-12 mAbs (a mixture of antibodies 4A1 and **20C2**, each at 10 .mu.g/ml).

DETDESC:

DETD(214)

Human . . . used as a control. Monoclonal anti-IL-12 antibody was a 1:1 mixture of two rat monoclonal anti-human IL-12 antibodies, 4A1 and **20C2**, which were isolated and purified as previously described (19). The 4A1 antibody is specific for the 40 kDa subunit of human IL-12, and its isotype is IgG2b. The **20C2** antibody appears to react with the 35 kDa subunit of IL-12, and its isotype is IgG1. These two antibodies were. . .

TITLE:	Monoclonal antibodies to cytotoxic lymphocyte maturation factor		
US PAT NO:	5,780,597 [IMAGE AVAILABLE]	DATE ISSUED:	Jul. 14, 1998
APPL-NO:	08/460,061	DATE FILED:	Jun. 2, 1995
REL-US-DATA:	Division of Ser. No. 205,011, Mar. 2, 1994, abandoned, which is a division of Ser. No. 857,023, Mar. 24, 1992, abandoned, which is a continuation-in-part of Ser. No. 572,284, Aug. 27, 1990, abandoned, which is a continuation-in-part of Ser. No. 520,935, May 9, 1990, abandoned, which is a continuation-in-part of Ser. No.		

receptor subunit simply binds **IL-12** heterodimer composed of covalently bound p35 and p40 chains with low affinity. Upon binding of IL-6/soluble IL-6R, gp130 dimerizes and. . . appears to be ligand independent, and a further receptor subunit is likely to be required to yield a high-affinity, functional **IL-12** receptor. In that context, it is interesting to note that a previous analysis of the **IL-12** receptor on PHA blasts identified an **IL-12** binding protein of about 110 KDa and a receptor associated protein of about 85 KDa (R. Chizzonite, et al., 1992,. . . the 110 KDa protein represents the subunit whose structure is reported here. It will be interesting to characterize the additional **IL-12** receptor component and evaluate its effects on **IL-12** binding and signal transduction.

TITLE:	Human receptor for interleukin-12		
US PAT NO:	5,831,007 [IMAGE AVAILABLE]	DATE ISSUED:	Nov. 3, 1998
APPL-NO:	08/419,652	DATE FILED:	Apr. 11, 1995
REL-US-DATA:	Division of Ser. No. 248,532, May 31, 1994, Pat. No. 5,536,657, which is a continuation-in-part of Ser. No. 94,713, Jul. 19, 1993, abandoned.		

US PAT NO: 5,650,492 [IMAGE AVAILABLE] L10: 6 of 7
TITLE: P-40 homodimer of interleukin-12

ABSTRACT:
Analysis of the culture media of p40-transfected COS cells indicated the presence of 40 kDa monomers and 80 kDa disulfide-linked homodimers. Examination of partially purified p40 recombinant proteins demonstrated that only the homodimer but not the monomer binds to the IL-12 receptor. Partially purified 80 kDa homodimer inhibited [.sup.125 I]IL-12 binding to PHA-activated human lymphoblasts with an IC.sub.50 of 80 ng/ml, which is similar to the IC.sub.50 value (20 ng/ml) for the human IL-12 heterodimer. Although neither the 40 kDa monomer nor the 80 kDa dimer could stimulate human PHA-blast proliferation, the 80 kDa dimer inhibited IL-12-induced proliferation in a dose-dependent manner with an IC.sub.50 of 1 .mu.g/ml. The IL-12 p40 subunit contains the essential epitopes for receptor binding, but they are only active when p40 is covalently associated with a second protein such as p35 or p40. When p40 is associated with the p35 subunit, the heterodimer acts as an agonist mediating biologic activity. When p40 associates with itself, the homodimer behaves as an antagonist.

ABSTRACT:
Analysis of the culture media of p40-transfected COS cells indicated the presence of 40 kDa monomers and 80 kDa disulfide-linked homodimers. Examination of partially purified p40 recombinant proteins demonstrated that only the homodimer but not the monomer binds to the IL-12 receptor. Partially purified 80 kDa homodimer inhibited [.sup.125 I]IL-12 binding to PHA-activated human lymphoblasts with an IC.sub.50 of 80 ng/ml, which is similar to the IC.sub.50 value (20 ng/ml) for the human IL-12 heterodimer. Although neither the 40 kDa monomer nor the 80 kDa dimer could stimulate human PHA-blast proliferation, the 80 kDa dimer inhibited IL-12-induced proliferation in a dose-dependent manner with an IC.sub.50 of 1 .mu.g/ml. The IL-12 p40 subunit contains the essential epitopes for receptor binding, but they are only active when p40 is covalently associated with. . .